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HEAT SHOCK-INDUCED CELL DEATH

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HEAT SHOCK-INDUCED CELL DEATH

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Dedication

To my dearest parents,

Long-Ji Chern & Shiu-Yuan Lai,

My brother,

Shun-Jia Chen Ph.D.,

Who have believed in me

Without your support, my success was just a dream

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It was early in the spring of 2009, when I went to talk to Dr. Shawn B. Bratton to ask about the possibility of volunteering in his lab. My previous studies had been in a completely different scientific field, so I wanted to learn new scientific techniques. He was very generous and kind to let me learn something new in his lab as a volunteer, and I enjoyed doing new types of experiments. It turned out that he became my supervisor for the next 7 years of my Ph.D. studies. I would like to express my gratitude to Shawn for his support, guidance, and advice throughout my entire Ph.D. Had he not offered me an opportunity at that time, I would not be here today. Thank you for always being patient and understanding with me even when I was going through a rough time. Thank you for believing in me, even when I was having doubts. Thank you for sharing with me your enthusiasm for science and teaching me how to be passionate about science. It has been a long journey, and I would not have made it this far without your support.

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HEAT SHOCK-INDUCED CELL DEATH

Miao-Der Chen, Ph.D.

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Hyperthermia or heat shock therapy has been utilized in clinical cancer therapy in combination with surgery, chemotherapy, or radiotherapy for the treatment of various types of cancer. However, the precise mechanisms that mediate heat shock-induced apoptotic signaling remains unclear. In previous studies, we have found that none of the known initiator caspases-1, -2, -4, -8, -9, -10, -12, or their caspase-activating complexes, is essential for promoting mitochondrial outer membrane permeabilization (MOMP) or activating caspase-3 following heat shock. Nevertheless, each of the aforementioned events could be significantly inhibited with the pan-caspase inhibitor Z-VAD-FMK. Pro-apoptotic BCL-2 family members Bax and Bak are essential for inducing MOMP and mediate apoptosis induced by most types of stressful stimuli. Remarkably, however, caspase-3 was still activated in $Bax^{-/-}/Bak^{-/-}$ DKO MEFs and about 50 % of cells still died following heat shock, even though they failed to undergo MOMP. This suggested that there is another protease, upstream of mitochondria that could activate caspase-3 following heat shock. In subsequent experiments, we discovered that heat shock induced lysosomal membrane permeabilization (LMP) as detected by loss of LysoTracker®-Green and release

of cathepsins. Cells lacking the BH3-only protein Bid, which is a direct activator of Bax/Bak, and often cleaved by lysosomal proteases, were not resistant to heat-induced LMP and cell death. However, cells lacking the BH3-only protein Bim were fully resistant to both LMP and cell death, indicating that both events were Bim-dependent.

Since heat shock induced the release of cathepsins into the cytosol and some cathepsins are inhibited by Z-VAD-FMK, we speculate that cathepsins might serve as the apical protease. In agreement of this hypothesis, overexpression of a cysteine cathepsin inhibitor suppressed cell death and knockout of cathepsin L completely blocked it. Thus, Bim mediates Bax/Bak-dependent and Bax/Bak-independent cell death following heat shock, and these pathways involved cathepsin L.

More recently, we discovered that Bim mutants (BimG154E and BimΔBH3), that could not activate Bax or Bak, nevertheless mediated heat shock-induced LMP and cell death. $Bim^{-/-}$ cells and those expressing a Bim mutant (BimG154E/STA) that could no longer associate with the dynein motor complex were fully resistant to heat shock. Thus, Bim's ability to induce heat shock-induced LMP and cell death did not require its BH3 domain but did require an interaction with LC8 light chain of dynein motor complex. It has been suggested that Bim is sequestered by LC8 in order to prevent unintentional apoptosis. Moreover, a recent study suggests that Bim and LC8 sequester Becin-1, a key regulator of autophagy. However, knockout of essential autophagy genes ATG5 and ATG16L1 did not significantly enhance cell death. Instead, we found the novel discovery that Bim's association with the dynein motor complex is important for regulating the trafficking of lysosomes. We

observed that loss of Bim resulted in a significant decrease in the number and position of lysosomes within the cell, and that reintroduction of Bim (G154E) or Bim (ΔBH3) but not Bim (G154E/STA), resulted in normal lysosome number and distribution.

Finally, changes in lysosomal distribution paralleled changes in the secretion of cathepsins into the extracellular space, and both intracellular and extracellular cathepsins appeared to participate in cell death. Thus, in summary, Bim mediates lysosomal cell death through its regulation of lysosome positioning and cathepsin trafficking.

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List of Abbreviation

APaf-1	Apoptotic protease-activating factor-1
AP20187	FKBP ligand (homodimerizer)
ATP	Adenosine triphosphate
Bad	Bcl-2 associated death promoter
Bak	Bcl-2 antagonist killer
Bax	Bcl-2-associated X protein
BCL-2	B-cell leukemia/lymphoma-2
BCL-X_L	B-cell lymphoma extra-large
BFL1/A1	Bcl-2 related protein A1
BH	Bcl-2 homology
BH3	Bcl-2 homology domain 3
Bid	BH3 interacting domain death agonist
Bik	Bcl-2-interacting killer
Bim	Bcl-2-interacting mediator of cell death
Bmf	Bcl-2-modifying factor
CARD	Caspase activation and recruitment domain
Cas9	CRISPR associated protein 9
Caspases	Cysteinyl aspartate-specific proteases
CtB	Cathepsin B

CtL	Cathepsin L
c-FLIP	Cellular-FLICE-like inhibitory protein
cIAP1/2	Cellular inhibitor of apoptosis-1/2
cyt. c	Cytochrome c
CRISPR	Clustered regularly interspaced short palindromic repeat
CSTB	Cystatin B/Stefin B
dATP	Deoxyadenosine triphosphate
DD	Death domain
DDR	DNA damage response
DED	Death effector domain
DISC	Death-inducing signaling complex
DKO	Double knockout
DLC-1	Dynein light chain -1
DMSO	Dimethyl sulfoxide
EE	Early endosome
ER	Endoplasmic reticulum
FADD	Fas-associated protein with death domain
FKBP	FK506-binding protein
FOXO3a	Forkhead box O3a
GFP	Green fluorescent protein
HRK	Harakiri

HS	Heat shock
HSC70	Heat shock cognate protein 70
HSP	Heat shock protein
HSP70	Heat shock protein 70
IAP	Inhibitor of apoptosis
JNK	c-Jun N-terminal kinase
KIF2A	Kinesin family member 2A
LC8	Dynein light chain subunit 8
LMP	Lysosomal membrane permeabilization
Leu-Leu-OMe	Leu-Leu methyl ester hydrobromide
LE	Late endosome
MAPK	Mitogen-activated protein kinases
M6P	Mannose 6-phosphate
M6PR	Receptor of Mannose 6-phosphate
MCL-1	Myeloid cell leukemia-1
MEF	Mouse embryonic fibroblast
MOMP	Mitochondrial outer membrane permeabilization
PARP	Mitochondrial outer membrane permeabilization
PBS	Phosphate-buffered saline
Pi	Propidium iodide
PI3K	Phosphatidylinositol (PI) 3-kinases

PS	Phosphatidylserine
PUMA	p53 up-regulated modulator of apoptosis
p38	p38 MAPK
RILP	Rab-interacting lysosomal protein
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNAi	RNA interference
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
shRNA	Small-hairpin RNA
Smac/DIABLO	Second mitochondria-derived activator of caspases/ direct IAP binding protein with low pI
Syt VII	synaptotagmin VII
tBid	Truncated bid
TBS	Tris-buffered saline solution
TGN	*Trans*-Golgi network
TMRE	Tetramethylrhodamine ethyl ester
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRADD	TNFR1-associated death domain
TRAF	TNF-receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand

UV	Ultraviolet radiation
UVRAG	UV radiation resistance-associated gene
Vec Ctrl	Vector control
WT	Wild-type
XIAP	X-linked IAP
Z-VAD-FMK	Z-valine-alanine-aspartate-fluoromethylketone
ΔΨm	Mitochondrial membrane potential

Chapter 1: Literature Review

1.1. Programmed cell death

All multicellular organisms require properly regulated cell death in order to maintain normal cell number and physiological functions of various tissues. Programmed cell death is required to eliminate those aged, damaged, or unhealthy cells inside the body and does so through execution of sophisticated genetically-controlled mechanisms. Although apoptosis is the best characterized form of programmed cell death, lysosomal-mediated cell death also plays an important role in mediating cell death in response to various stimuli and, in many cases, integrates with classical apoptotic signaling pathways[1-4]. Apoptosis is controlled by diverse cell signaling pathways and can be subcategorized into either “extrinsic” or “intrinsic” pathways, depending upon the type of stressful stimulus. Lysosomal-mediated cell death requires lysosomal membrane permeabilization (LMP)[5-7] and involves the release of hydrolytic enzymes, including cathepsin proteases[2-4, 7-12]. Apoptotic cell death pathways, including those that involve LMP, are important in regulating normal physiological functions and in maintaining the normal homeostasis[13]. Endogenous cathepsin inhibitors, such as cystatin B (CSTB), are important in regulating lysosomal-mediated cell death, and loss of CSTB appears to increase apoptosis in cerebellar granule neurons during progressive myoclonic epilepsy[14, 15], implying that this endogenous cathepsin inhibitor is required to prevent excessive lysosomal-

mediated cell death in *vivo*. Indeed, loss of homeostatic balance between cell proliferation and cell death, including excessive or insufficient of lysosomal-mediated cell death, contributes to many diseases, including tumorigenesis[13, 16-19]. Previous reports suggest that cathepsins can serve as either tumor promoters or tumor suppressors, depending upon the context. For example, intracellular release of cysteine cathepsins participates in the killing of cancer cells following irradiation and various forms of chemotherapy; however, increased secretion of cathepsins into the extracellular space can also contribute to the break down of extracellular matrix, stimulating angiogenesis, migration, and metastasis[20, 21].

1.2. Apoptosis

Apoptosis is derived from an ancient Greek word that means “leaves falling from a tree.” In 1972, Kerr, Wyllie, and Currie first used the word “apoptosis” in a paper published in the *British Journal of Cancer* [22]. Apoptosis is described as a specific form of programmed cell death, characterized by morphological changes such as rounding-up, shrinkage or pyknosis, nuclear fragmentation, chromatin condensation, and plasma membrane blebbing, among others [23]. Biochemically, during apoptosis, redistribution of phosphatidylserine from the inner to the outer leaflet of the plasma membrane provides “eat me” signals to resident phagocytic cells for their removal. This process is in stark

contrast to necrosis, where intracellular contents are released into the extracellular space and trigger inflammation[24]. The process of apoptosis is initiated and controlled by diverse cellular signaling pathways, which may originate from either an extracellular (extrinsic) or an intracellular (intrinsic) stimulus. Another biochemical hallmark of apoptosis is the activation of cysteinyl aspartate-specific proteases (caspases), which are expressed as single-chain inactive proteases, or "zymogens"[24]. Most of these proteases are present in the cytoplasm and can be sub-divided into two categories: initiator caspases, which are at the top of the cell death cascade; and effector caspases, which are activated by initiator caspases. Once activated through proteolytic processing, effector caspases can cleave >1,000 cellular substrates, resulting in the prototypical morphological changes observed during apoptosis.

1.2.1. Extrinsic pathway

The extrinsic pathway is typically initiated by death ligands of the tumor necrosis factor (TNF) superfamily, which stimulate cell surface death receptors (DRs) and activate downstream intracellular apoptotic pathways (Figure 1.1). These ligands possess soluble extracellular transmembrane domains, which can remain associated with the membranes of effector cells (e.g. cytotoxic T cells) and mediate cell death through interactions with target cells; or they can be cleaved at the cell surface by metalloproteinases and released as soluble ligands. These ligands, which are generally trimeric, bind to their cognate TNF

receptor (TNFR) family members, including TNFR1, Fas (as known as CD95), and TRAIL receptors, DR4 and DR5, which in turn induce receptor trimerization and activation. TNFR1, Fas, and DR4/DR5 possess intracellular death domains (DDs), which recruit other DD-containing proteins, such as TNFR1-associated death domain (TRADD) and Fas-associated protein with death domain (FADD)[25, 26]. FADD, in turn, binds to the initiator caspases-8 and -10, facilitating proximity-induced homodimerization, intermolecular processing, and activation. These receptor complexes are frequently referred to as *death inducing signaling complexes* (DISCs). Following caspase-8 activation, it generally processes the downstream effector caspases-3 and -7. However, caspase-8 also cleaves the BH3-only BCL-2 family member Bid, resulting in the formation of truncated Bid (tBid), which promotes the activation multidomain pro-apoptotic BCL-2 family members, namely BCL-2-associated X protein (Bax) and/or BCL-2 antagonist or killer (Bak)[27]. Oligomerization and insertion of Bax/Bak into the outer mitochondrial membrane (OMM) permeabilizes it and allows for the release of cytochrome c from the intermembrane space into the cytosol. Cytochrome c then initiates activation of the intrinsic or mitochondrial cell death pathway.

1.2.2. Intrinsic pathway

The Intrinsic pathway is initiated in response to stressful stimuli, such as nutrient deprivation, DNA damage, ER stress, viral infection, etc. As already noted, the intrinsic or mitochondrial pathway is characterized by mitochondrial

outer membrane permeabilization (MOMP) and the release of cytochrome c, along with other apoptogenic proteins including Smac/Diablo, endonuclease G, Omi/HtrA2, and AIF, into the cytosol (Figure 1.1). Once released, cytochrome c stimulates (d)ATP-dependent oligomerization of the adapter protein, apoptosis protease-activating factor 1 (Apaf1), into an "apoptosome" complex that sequentially activates the initiator caspase-9 and the effector caspases-3 and -7. Smac/Diablo and Omi/HtrA2 potentiate apoptosome activity by antagonizing X-linked inhibitor of apoptosis (XIAP), which inhibits the catalytic activities of caspases-9, -3, and -7[26, 28].

1.2.3. BCL-2 Family members

The B cell lymphoma-2 (BCL-2) family is one of the more well-characterized protein families involved in the regulation of apoptotic cell death. They primarily regulate MOMP and the release of cytochrome c as well as other apoptogenic proteins. The regulation of apoptosis by BCL-2 family members is crucial for normal tissue homeostasis and embryonic development, and consequently, alterations in their expression levels or defects in their function can impact various diseases, ranging from neurodegeneration to cancer[7, 29, 30]. Both pro-apoptotic and anti-apoptotic BCL-2 proteins share regions of structural homology, known as BCL-2 homology (BH) domains, and in some cases, possess C-terminal transmembrane domains (Figure 1.2)[31]. BCL-2 family members can be subdivided into three categories: anti-apoptotic family members (BCL-2, BCL-X_L, BCL-W, A1 and MCL-1), which typically contain BH1 through

BH4 domains; multidomain pro-apoptotic family members (Bax, Bak, and Bok), which contain BH1-BH3 domains; and BH3–only pro-apoptotic family members (Bim, Bid, Bad, Bmf, Puma, and Noxa, among others), which possess only BH3 domains and display no homology outside of this region. These BH3-only proteins are thought to be essential for stress-induced apoptosis and thus act by inhibiting antiapoptotic BCL-2 family members and promoting Bax/Bak-mediated MOMP and the release of apoptogenic proteins.

1.3. BH3-only proteins in apoptosis

As alluded to above, BH3-only family members are key players in canonical intrinsic or mitochondrial pathway. They mediate their effects through activation of pro-apoptotic Bax/Bak and/or inhibition of anti-apoptotic family members, such as BCL-2, MCL-1, and BCL-X_L (Figure 1.3). In the "indirect activation model", BH3-only proteins inhibit anti-apoptotic BCL-2 family members, relieving their inhibition of Bax/Bak. Removal of this brake on Bax and Bak promotes their spontaneous homo-oligomerization in the OMM, resulting in MOMP. In the "direct activation model", *activator* BH3-only proteins, such as Bim, Bid, and Puma, bind directly to Bax or Bak and induce conformational changes in these proteins necessary for oligomerization and MOMP [32, 33]. By contrast, *sensitizer* BH3-only family members, such as Bad, Noxa, and Bik, bind

to anti-apoptotic family members and displace the activator BH3-only family members, allowing them to directly activate Bax or Bak.

1.3.1. BCL-2 interacting mediator of cell death (Bim)

Bim was originally identified by two separate groups in yeast two-hybrid screens, where it was given the names Bim and Bod (BCL-2 related ovarian death agonist)[34, 35]. The BH3 domain of Bim and its C-terminal hydrophobic region are reportedly important for its pro-apoptotic function, and Bim has been implicated in cell death initiated by several apoptotic stimuli, including cytokine withdrawal[36], DNA damaging agents (γ irradiation, doxorubicin, UV)[36], ER stress[37], cytoskeletal damage[38], and anoikis[38, 39]. Bim and Bid have also been implicated in various apoptotic-signaling pathways that result in lysosomal membrane permeabilization (LMP). Indeed, JNK phosphorylation of Bim has been placed upstream of LMP and cell death in UVB-induced apoptosis of human melanocytes[40] and TRAIL-induced apoptosis[41]. TRAIL induces recruitment of the multifunctional sorting protein, phosphofurin acidic cluster sorting protein-2 (PACS-2), to the endosomal compartment along with Bim and Bax[42]. Indeed, recombinant PACS-2 also binds to Bim in *vitro*, which suggests that Bim may play an important role in regulating TRAIL-induced LMP and cell death[42]. As previously noted, Bim not only contains a BH3 domain but also a C-terminal hydrophobic region; therefore, Bim may interact with lysosomal membranes *via* its C-terminus. Precisely how Bim regulates LMP remains unclear, but these studies provide a framework with which to investigate Bim-

mediated LMP. Importantly, Bim is also reportedly deleted or epigenetically-silenced in various cancers, including leukemia, colon cancer, and lung cancer, and loss of Bim expression correlates with therapeutic resistance. Moreover, Bim mimetics (*e.g.* ABT-263) and lysosomotropic agents effectively increase the sensitivity of many resistant cancer cells to the chemotherapy.

BH3-only proteins are regulated *via* both transcriptional and post-translational mechanisms. Given the extent of its transcriptional and post-translational regulation, it is perhaps not surprising that Bim knockout mice exhibit prominent lymphoid hyperplasia, indicating that it plays a role in mediating normal lymphoid homeostasis[43, 44]. There are three splice variants of Bim (Bim_s, Bim_L, and Bim_{EL}), with Bim_s being the most potent killer, and transcription of Bim is regulated by the forkhead transcription factor, FOXO3a[45], E2F1[46], CHOP-C/EBP[47], RelA, glucocorticoid receptor[48], and the AP-1 family member c-Jun[49]. Yeast two-hybrid studies and immunoprecipitation analyses have also demonstrated that Bim_L and Bim_{EL} bind to the cytoplasmic dynein light chain, LC8 (also known as DLC1)[50]. LC8 is a component of the microtubule-associated dynein motor complex; therefore, previous reports suggest that LC8 sequesters Bim and suppresses spontaneous apoptosis. Interestingly, however, phosphoryl-ation of Bim at Thr112 by c-Jun N-terminal kinases (JNKs) disrupts Bim's interaction with LC8 and allows it to translocate to mitochondria, where it can interact with BCL-2 family members[51]. More recently, Rubinzstein and

colleagues have revealed a non-apoptotic function of the Bim-LC8 interaction. The complex apparently also recruits and sequesters Beclin-1, which is a key positive regulator of autophagy[52, 53]. Beclin-1 is an allosteric activator of the PI3 kinase VPS34, which is essential for stimulating the formation of autophagosomes. Therefore, sequestration of Beclin-1 and Bim by LC8 appears to simultaneously suppress cell death and autophagy[34, 52-55].

1.3.2. BH3 interacting-domain death agonist (Bid)

Bid was first identified in 1996 using interactive cloning of a cDNA library with BCL-2 and Bax, and it was shown to induce caspase-dependent cell death *via* its BH3 domain[56]. Full-length Bid is expressed in most tissues; however, it remains relatively inactive until proteolytically cleaved. Bid can be cleaved by caspase-8, and once processed, truncated Bid (tBid) activates Bax/Bak and induces oligomerization and MOMP. Therefore, Bid cleavage links the extrinsic and intrinsic apoptotic pathways together following death receptor signaling. Notably, cathepsins also engage the mitochondrial cell death pathway through Bid cleavage[6, 57, 58]. Following LMP, cathepsins D, B, and L may cleave Bid, at a site near its caspase cleavage site, thereby engaging the mitochondrial cell death pathway and enhancing cell death.

In the context of heat shock-induced apoptosis, Green and colleagues have previously proposed that heat shock induces cell death through activation

of caspase-2, which can also cleave Bid but does so rather inefficiently compared to caspase-8[59, 60]. Regardless, they argue that tBid then activates Bax/Bak, induces MOMP, and stimulates apoptosome-dependent activation of effector caspases. In their study, loss of either caspase-2 or Bid inhibited heat shock-induced cell death, which suggested that caspase-2 and Bid were both essential for heat shock-induced cell death. However, this model remains controversial, as we (and others) have previously found that caspase-2 and Bid contribute very little to cell death and are unnecessary at higher doses of heat shock[61-63]. This suggests that, at best, caspase-2-dependent cleavage of Bid serves only to amplify the apoptotic signal following a weak stimulus.

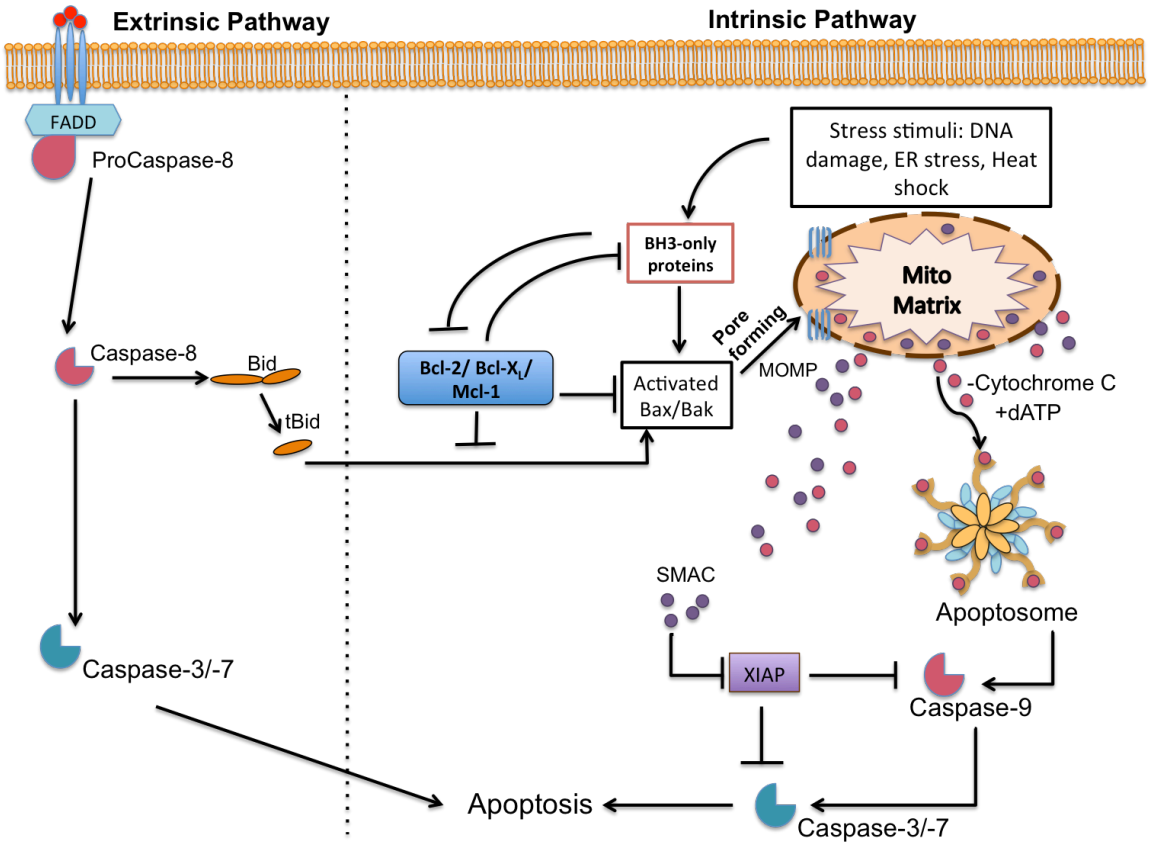

Figure 1.1. Intrinsic and Extrinsic apoptotic pathways.
Apoptosis can be induced by cell surface receptors, such as Fas and TNFR1 (extrinsic pathway, left), or by various types of stressful stimuli (intrinsic pathway). The intrinsic pathway often begins with BH3-only protein induction or post-translational activation, which results in the inactivation of some BCL-2 family members. Some BH3-only proteins, such of Bim and Puma, may also directly activate Bax/Bak, induce MOMP, and stimulate cytochrome c release, resulting in apoptosome formation and activation of caspases-9 and -3. In the extrinsic pathway, activated caspase-8 can directly activate caspase-3 and induce cell death. However, in some resistant cells, cleavage of Bid is necessary to activate the intrinsic pathway and amplify the cell death signal. Adapted from Ref.[64]

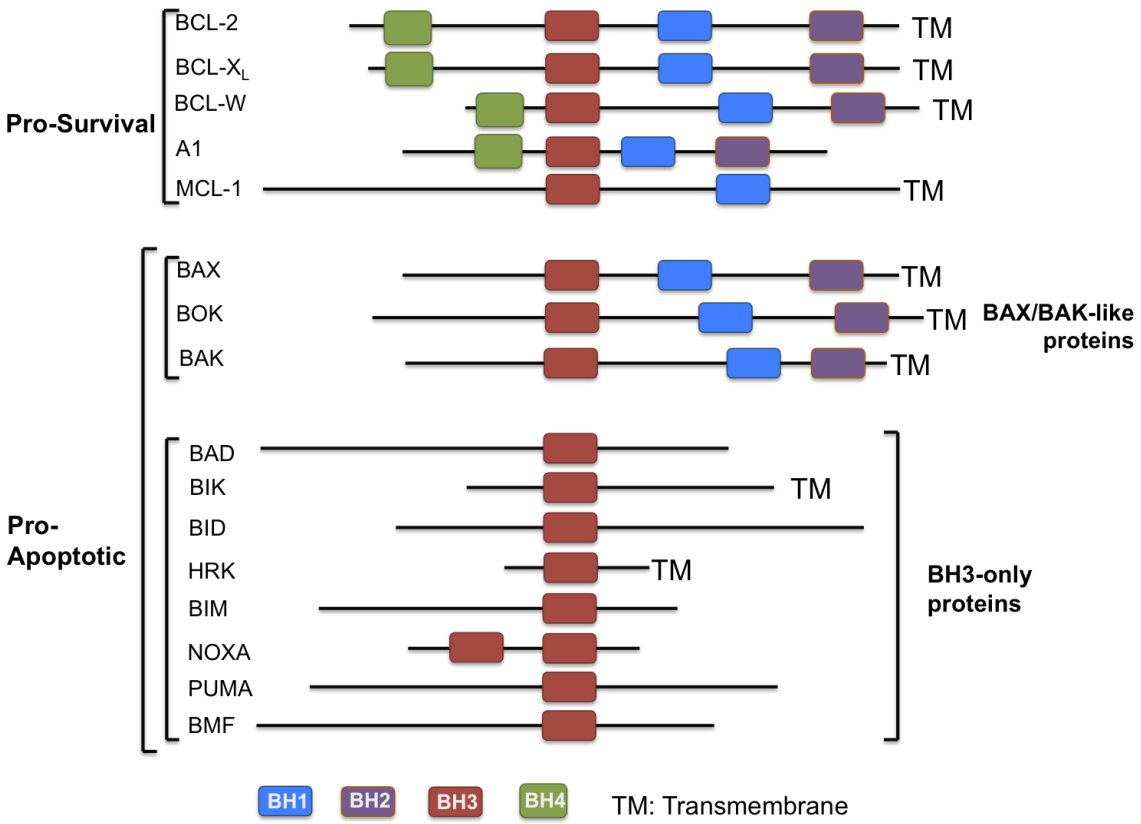

Figure 1.2. The mammalian BCL-2 family members.
A diagrammatic representation of the mammalian BCL-2 family members is shown. The pro-survival family members, BCL-2, BCL-X_L, BCL-W, A1, and MCL-1 (myeloid-cell leukemia sequence 1) possess 2-4 distinct BCL-2 homology (BH) domains. The pro-apoptotic members of the BCL-2 family can be subdivided into two groups: the multidomain proapoptotic family members, BAX, BAK, and BOK (BCL-2 related ovarian killer), which contain three distinct BH domains; and the BH3-only proteins, BAD (BCL-2 antagonist of cell death), BIK (BCL-2-interacting killer), BID (BH3-interacting-domain death agonist), HRK (harakiri), BIM (BCL-2 interacting mediator of cell death), NOXA, PUMA (p53-upregulated modulator of apoptosis), and BMF (BCL-2 modifying factor), which share only a conserved BH3 domain. Adapted from Ref. [29]

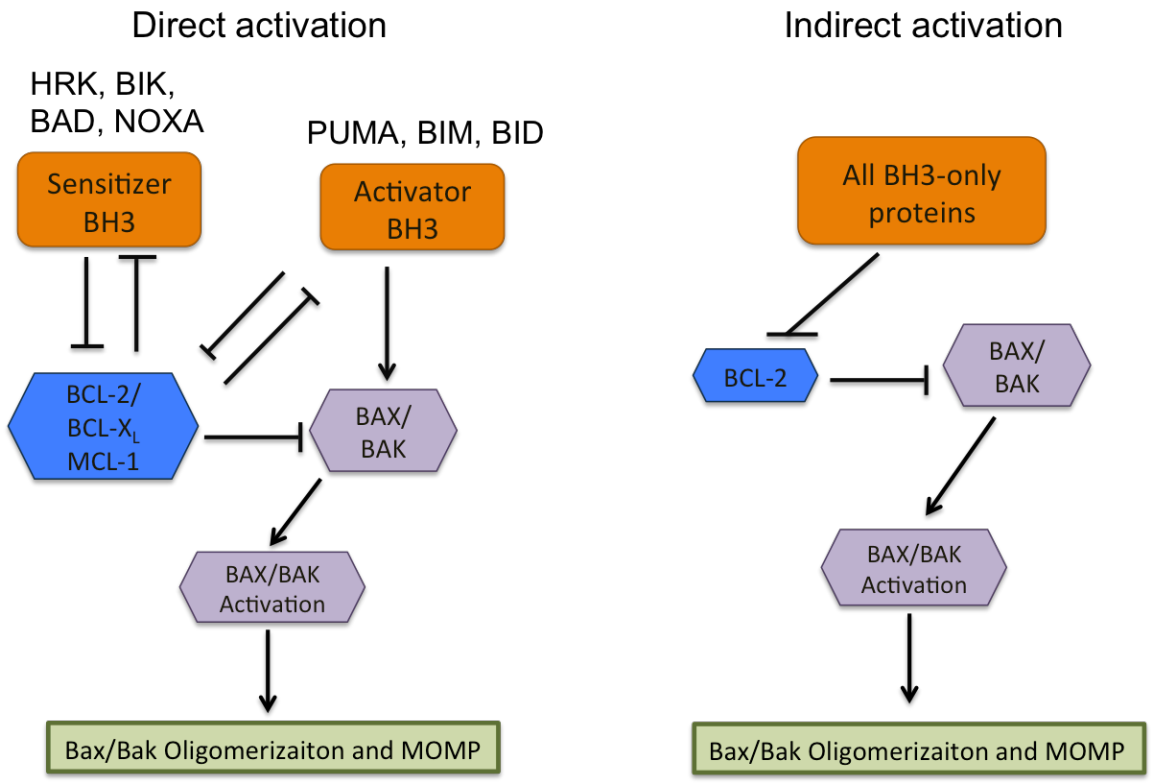

Figure 1.3. Models of Bax/Bak activation.
There are two proposed models to explain how BCL-2 family members regulate MOMP. In the direct activation model, the "activator" BH3-only proteins, tBid, Bim and Puma, directly engage and activate the pro-apoptotic effectors Bax and Bak. Pro-survival proteins, such as BCL-2, sequester BH3-only proteins and inhibit BAX/BAK. The "sensitizer" BH3-only proteins, such as BAD, NOXA and BIK, do not directly activate BAX or BAK, but instead displace the direct activators from anti-apoptotic family members, which can in turn activate BAX and BAK. Adapted from Ref. [65]

1.4. The Lysosome

The term “lysosome” is a Greek word meaning “digestive body”. Adhering to its own meaning, lysosomes are the cell’s degradation center, which is primarily responsible for the breakdown of proteins, polysaccharides, and complex lipids into amino acids, monosaccharides, and free fatty acids[18, 66, 67]. Lysosomes contain >60 hydrolytic enzymes, including proteases, glycosidases, sulfatases, phospholipases, phosphatases, and lipases, which degrade intracellular and extracellular materials following the encapsulation of intracellular cargo into autophagosomes (autophagy) or the internalization of extracellular cargo into endosomes, both of which eventually fuse with lysosomes. Most of these enzymes have their maximal enzymatic activity at low pH (pH≤5), and the acidic milieu of the lysosome is maintained *via* the vacuolar ATPase (V-ATPase), which pumps protons from the cytoplasm into the lumen[17]. Lysosomal membrane proteins, LAMP-1 and LAMP-2, are highly glycosylated membrane proteins that protect the lysosomal membrane from degradation by acidic enzymes and agents that might otherwise compromise the lysosomal membrane[17, 18, 68, 69].

Lysosome-mediated catabolic degradation is an adaptive process regulated by nutrient status and cellular signaling. Indeed, an increase in endocytosis and autophagy, followed by lysosomal degradation, results in the accumulation of catabolites, such as amino acids, which in turn downregulate

degradation and autophagic flux. In order to adapt to the changing cellular environment, lysosomes contain a nutrient-sensing mechanism that consists of the serine/threonine kinase, mammalian target of rapamycin (mTOR), and its associated proteins. Nutrient starvation not only inhibits mTOR-mediated growth, but also increases autophagosome formation. Thus, lysosomal dysfunction can lead to many serious medical conditions. Lysosomal storage diseases (LSDs) are thought to be caused by defects in degradation, catabolite export, or trafficking. Moreover, the impaired function of lysosomal channels contributes to multiple LSDs, and dysfunctional lysosomes have been linked to an imbalance in the regulation of autophagy and tumorigenesis[17, 70].

1.4.1. Lysosomes and Endocytosis

One of the major functions of lysosomes is to digest the material taken up from outside the cell by endocytosis, and the formation of lysosomes depends in part on endocytosis. Clathrin-coated endocytic vesicles initially bud from the plasma membrane and fuse with early endosomes. Some membrane components are recycled back to the plasma membrane, but these early endosomes gradually mature into late endosomes. One of the important changes during endosome maturation is the lowering of the internal pH to ~5.5, which plays a key role in the delivery of lysosomal acid hydrolases from the Golgi network. In mammalian cells, many newly synthesized hydrolases, including cathepsins, are post-translationally modified by glycosylation and further tagged

with mannose-6-phosphate (M6P) in the *cis*-Golgi and subsequently recognized by M6P receptors in the *trans*-Golgi network (TGN)[71]. Transport vesicles then deliver these M6P-tagged acid hydrolases to early endosomes, where they are subsequently sorted to late endosomes and dissociate from M6P receptors as a result of lumenal acidification. The M6P receptors are recycled back to the Golgi[71-74], whereas free procathepsins undergo cleavage to remove their inhibitory propeptides[73]. These propeptides are either cleaved by other proteases or autocatalytically removed at low pH, ultimately residing in the late endosomes and lysosomes[73-75]. While a significant portion of cathepsins are sorted into lysosomes, as described, some transport vesicles fail to fuse with endosomes and instead fuse with the plasma membrane, delivering catalytically-inactive procathepsins into the extracellular space. Mature cathepsins can also be secreted into the extracellular space through lysosomal exocytosis, which is upregulated in response to plasma membrane damage and plays a major role in membrane repair. The latter process is regulated by synaptotagmin VII (Syt VII), which is a Ca^{2+} sensor and a regulator of exocytosis[76-80].

Microtubules originate from a microtubule-organizing center (MTOC), which is the major site of microtubule nucleation inside the cell. Many intracellular compartments, including lysosomes move along microtubules in a bidirectional manner. In this tightly regulated process, kinesin motor complexes move along microtubules toward the plus-end, away from the MTOC (cell

periphery), whereas the dynein motor complexes move toward the minus-end, near the MTOC (perinuclear region)[81, 82]. These complexes bind to microtubules *via* their heavy chains, which possess ATPase activity essential for motor activity, and to lysosomes through their light chains, which collectively regulate lysosomal transport and positioning[81, 83-85]. The small GTPase Arl8 and the kinesin regulator KIF2A also participate in regulating the localization of lysosomes[82, 84, 86]. Dynein light chain, on the other hand, regulates lysosomal movement through the adapter protein, Rab7-interacting lysosomal protein (RILP), and the small GTPase, Rab7, which is known to play an essential role in the fusion of lysosomes to late endosomes and autophagosomes[85, 87-89]. As already noted, the BH3-only protein Bim binds to LC8 and thus interacts with the dynein motor complex. While current dogma suggests that this interaction is solely for the purpose of sequestering Bim in order to prevent Bim-mediated apoptosis[28, 90], or to aid in the sequestration of Beclin 1 in order to prevent autophagy[52, 53, 55], it remains unclear whether Bim has a direct role in regulating lysosomal biogenesis, positioning, or exocytosis.

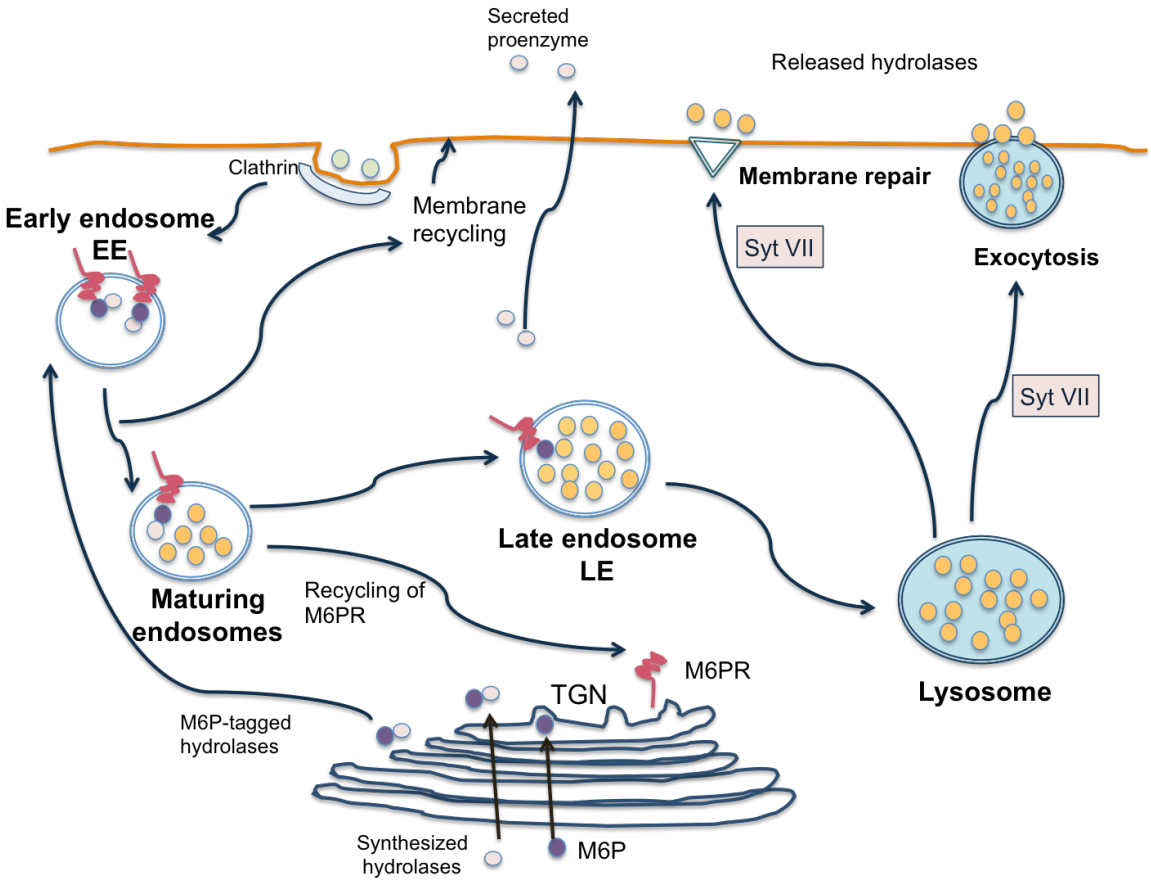

Figure 1.4. Endocytic lysosome pathways.
Endocytic cargo is internalized from the plasma membrane first to early endosomes and then to late endosomes. The newly sythesized hydrolases are modified with M6P, bind to M6P receptors, and are eventually sorted from the TGN to early endosomes. As early endosomes mature into late endosomes, procathepsins are processed as a result of lumenal acidification. Some transport vesicles fail to fuse with endosomes and instead fuse with the plasma membrane, delivering catalytically-inactive procathepsins into the extracellular space. However, mature cathepsins can also secreted as a result of lysosomal exocytosis, which is regulated by synaptotagmin VII (Syt VII) and mediates repair of damaged plasma membranes. Adapted from ref. [3, 91]

1.4.2. Lysosomes and Autophagy

There are three defined types of autophagy: macro-autophagy, micro-autophagy, and chaperone-mediated autophagy, all of which promote proteolytic degradation of cytosolic components within the lysosome[18]. Macro-autophagy delivers cytoplasmic cargo to the lysosome through the intermediary of double membrane-bound vesicles, referred to as autophagosomes, which fuse with lysosomes to form autolysosomes. In contrast, in micro-autophagy, cytosolic components are taken up directly by lysosomes through invagination of lysosomal membranes. Both macro and micro-autophagy are able to engulf large structures through both selective and non-selective mechanisms[92]. In chaperone-mediated autophagy, targeted proteins are translocated across lysosomal membranes, in complex with HSC70[93]. This chaperone and the targeted protein are recognized by the lysosomal membrane receptor, lysosomal-associated membrane protein 2A (LAMP-2A), resulting in its unfolding, transport across the lysosomal membrane, and degradation within the lysosomal lumen. Thus, lysosomes play an important role in normal autophagy.

Notably, lysosomal and autophagic alterations are common in cancer cells. Excessive autophagy reportedly plays a role in cancer resistance, and increased expression and altered trafficking of lysosomal enzymes participates in tissue invasion and angiogenesis. Lysosomes in cancer cells, however, are also more sensitive to permeabilization. Thus, heat shock protein 70 (HSP70), which

stabilizes lysosome membrane, is often upregulated in cancer and effectively prevents LMP. Indeed, resistance to several cancer therapeutics can be attributed, at least in part, to increased expression of HSP70[94-96]. Alterations in autophagy are similarly linked to carcinogenesis and resistance to chemotherapy [97]. Therefore, targeting lysosomes has enormous potential in the treatment of cancer as it can simultaneously promote cathepsin-mediated cell death while suppressing autophagy. Indeed, numerous studies have shown that lysosomotropic agents effectively sensitize resistant cancer cells to chemotherapy, resulting in better treatment outcomes[8, 12, 68, 98].

1.4.3. Cathepsins and their roles in apoptosis.

LMP, followed by release of cathepsins, represents an early and essential step in lysosomal-mediated cell death. There are agents reported to induce LMP, including death ligands such as TRAIL[41, 42, 99], TNF[100], and Fas ligand[101]; DNA damaging agents such as adriamycin and etoposide[102]; reactive oxygen species (ROS)[103]; or lysosomotropic agents such as Leu-Leu-OMe[104]. Lysosomes contain >50 acid hydrolases, including proteases, phosphatases, nucleases, glycosidases, sulphatases, and lipases, that aid in the degradation of intracellular and extracellular material. Among the hydrolases, cathepsins are a group of lysosomal proteases that play major roles in cleaving and/or degrading cellular proteins, especially during cell death[3, 12]. Cathepsins can be divided into three categories based on their active-site residues: cysteine, serine, and

aspartic acid proteases. Cysteine cathepsins constitute the largest group with 11 members, including cathepsins B, C, F, H, K, L, O, S, V, X, and W. Aspartic acid cathepsins include cathepsins D and E; and serine cathepsins include cathepsins A and G[8, 9].

Several studies have shown that cathepsins play important roles in apoptosis (Figure 1.5). Based upon the cell-type and cellular stress, cathepsins can either initiate or amplify the apoptotic signal. Some studies suggest that cathepsins, such as cathepsin B, are capable of cleaving caspase-3 directly[105]. Moreover, one or more of the cysteine cathepsins B, L, S, K, and H can cleave Bid, Bcl-2, Bcl-X_L, MCL-1, Bak, and BimEL following treatment of cells with the lysosomotropic agent, Leu-Leu-OMe[106]. Other than cysteine cathepsins, cathepsins D can cleave caspse-8 and participate in its activation, initiating neutrophil apoptosis and staurosporine-induced cell death in human fibroblast[107, 108]. Finally, cathepsins may act on neighboring lysosomes to permeabilize additional membranes, thereby propagating the further release of cathepsins. Once released, these cathepsins eventually induce either necrosis and/or apoptosis. Generally speaking, weaker insults that trigger limited LMP (and modest release of lysosomal proteases) induce apoptosis; whereas, strong insults induce massive LMP, complete release of lysosomal contents, and frank necrosis[3, 109, 110]. However, it is worth noting that, despite the many studies on lysosomal-mediated cell death, virtually nothing is known about the role(s) of

cathepsins in heat shock-induced cell death. Therefore, in this dissertation, the importance of cathepsins in heat shock-induced apoptosis will be investigated.

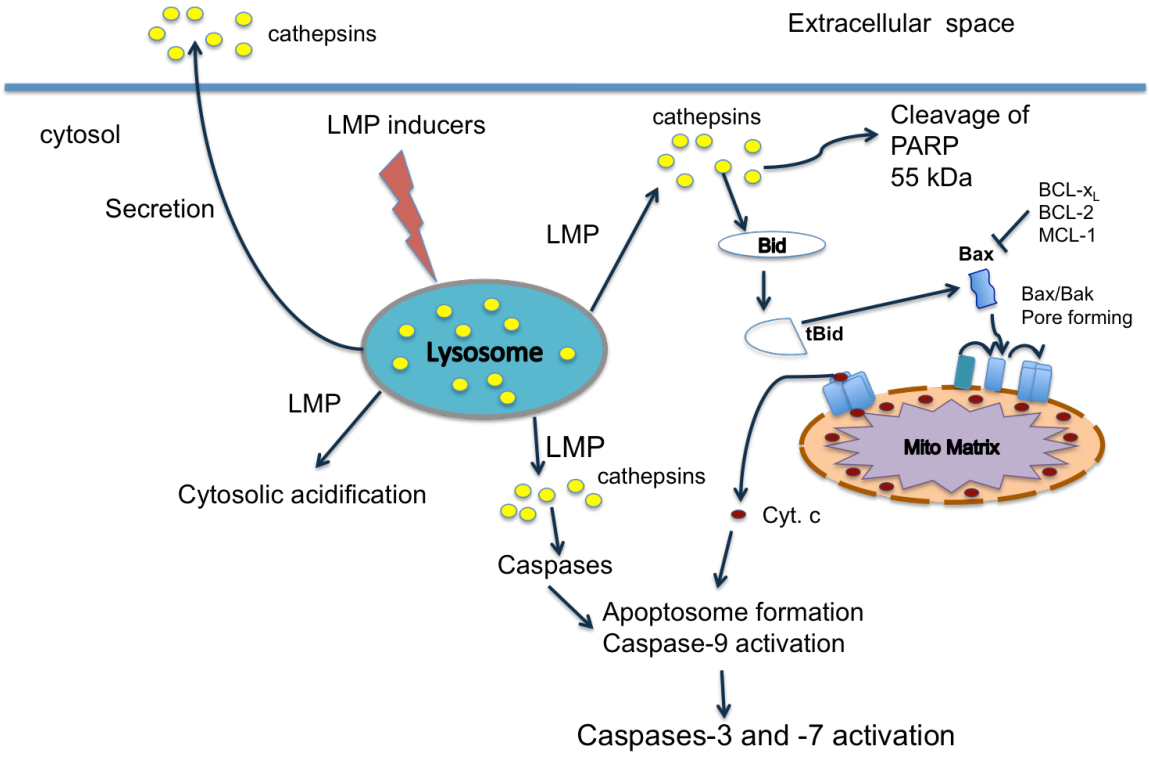

Figure 1.5. Integration of cathepsins into the intrinsic/mitochondrial pathway.
Following LMP, cathepsins are released into the cytosol where they can cleave the BH3-only protein, Bid. Trauncated Bid (tBid) then activates Bax/Bak, resulting in MOMP, cytochrome c release, and formation of the Apaf-1-caspase-9 apoptosome. Some cathepsins, such as cathepsin B, D, and L, may also directly activate caspases. Cathepsins also cleave PARP protein which can facilitate both apoptotic and necrotic cell death [109, 110].

1.5. Heat Shock-induced Cell Death

Hyperthermia, also known as heat shock, is an ancient stressful stimulus that has been widely applied to cancer therapy since the 1960s[111, 112]. Hyperthermia, as a cancer therapy, is based on the idea that targeted heating of a tumor can lead to cell death without injuring neighboring normal cells[113, 114]. Indeed, hyperthermic treatment can be given either locally, regionally, or to the whole body[113]. Unfortunately, hyperthermia alone has thus far largely failed in the clinic. The overall response rate is ~13% with many patients developing complications; there are difficulties in controlling local delivery to the tumor; and development of treatment resistance is common[113]. Recently, however, technological advances in the use of gold and magnetic nanoparticles[103, 27, 115, 116], non-invasive ultrasound[117], and laser-induced thermotherapy have made precise delivery of more intense heat more practical[118]. Although it can be applied, either alone or in combination with other cancer treatments, it is more effective when applied in combination with radio- or chemotherapy[94, 113, 119]. Most surprisingly, despite promising results in the treatment of some cancers and improvements in the delivery of heat, the actual cell death mechanism(s) induced by heat shock remain unclear.

1. 6. Study hypothesis and aims

During tumor development, cells undergo genetic modifications in order to escape apoptosis and support their malignant status. Furthermore, tumors often acquire resistance to cancer therapy and induction of LMP has recently been reported to re-sensitize tumor cells resistant to radio- and chemotherapy[120]. Previously, Green and colleagues have suggested that heat shock-induced cell death occurs through activation of caspse-2 and cleavage of Bid[59]. However, our previous work has shown that none of the known initiator caspases are essential for heat shock-induced cell death[62]. Moreover, though additional literature suggests that lysosomal-mediated cell death requires Bid and/or Bax/Bak to engage the mitochondrial pathway and induce cell death[1, 4-6, 10, 57, 58, 121], we have observed that heat shock induces LMP, caspase activation, and cell death in a Bim-dependent manner, independent of MOMP[63]. In short, despite many publications related to heat shock and lysosomal-mediated cell death, few, if any, have linked hyperthermia to cathepsin-mediated cell death. Therefore, the main goal for this dissertation was to identify and characterize the actual mechanisms whereby heat shock utilizes Bim to induce LMP (chapter #4) and to determine the role(s) that cathepsins play in mediating heat shock-induced cell death (chapter #3). We hypothesized that heat shock induces LMP, upstream of MOMP, through a Bim-dependent mechanism, and that cathepsins, released

following LMP, kill the cell through cleavage of critical substrates including caspase-3.

Chapter 2: Materials and Methods

2.1. Antibodies and Reagents

The following antibodies were purchased from Cell Signaling Technology (Danvers, MA): BAX (CS 2772); BAK (CS 3814); BIM (CS 2933); BID (CS 2003); BECLIN1 (CS 3495); SQSTM1/p62 (CS 5114); PI3 Kinases Class III VPS34 (CS 4263); LC3B (CS 2775); Caspase-3 (CS 9662); Cleaved Caspase-3 (CS 9661); Cleaved PARP (CS 9548); Cytochrome c (CS 11940); and COX IV (CS 4850). Cathepsins antibodies were purchased from R&D System (Minneapolis, MN): mCathepsin B (AF965); mCathepsin D (AF1029); h/mCathepsin L (MAB9521). Anti-Flag® M2 MA Affinity Gel (Cat. No. A2220) and monoclonal ANTI-FLAG® M2 antibody (Cat. No. F3165) were purchased from Sigma-Aldrich, St. Louis, MO. Cystatin B (Cat. No. 10823-1-AP) werepurchased from Proteintech™, Rosemont, IL.

OmniCathepsin®-fluorogenic substrate, Z-FR-AMC (Cat. No. BML-P139-0010) was purchased from Enzo Life Sciences, Farmingdale, NY, and digitonin (Cat. No. D141) was purchased from Sigma-Aldrich, St. Louis, MO. Fetal Bovine Serum (Cat. No. F0926) was obtained from Sigma-Aldrich, St. Louis, MO, and Dulbecco's Modification of Eagle's Medium (DMEM) (Cat. No. 50-003-PC) was purchased from CORNING, Manassas, VA. Ciliobreven-D (Cat. No. 250401) was purchased from EMD Millipore Calbiochem.

2.2. Plasmids

The following primers were used to clone various cDNA into the BamHI/NotI sites of the lentiviral expression vector FG9-EF1α-puro.

mCathepsin B US:	5’ CGG GAT CCA CCA TGT GGT GGT CCT TGA TCC TTC 3’
mCathepsin B DS:	5’ AAT GCG GCC GCT CAA GCA TAA TCT GGA ACA TCA TAT GGA TAG CTA GCG AAT CTT CCC CAG 3’
mCathepsin L US:	5’ CGG GAT CCA CCA TGA ATC TTT TAC TCC TTT TGG CT 3’
mCathepsin L DS:	5’ AAT GCG GCC GCT CAA GCA TAA TCT GGA ACA TCA TAT GGA TAG CTA GCA TTC ACG ACA GGA 3’
hCystatin B US:	5’ CGG GAT CCA CCA TGA TGT GCG GGG CGC CCT3’
hCystatin B DS:	5’ TTT TCC TTT TGC GGC CGC GAA ATA GGT CAG CTC ATC ATG C 3’
CystatinB ΔMMGC-US:	5’ CGG GAT CCA CCA TGG CGC CCT CCG CCA CGC AG 3’
hCystatinB L73G US:	5’CGA GTG TTC CAA TCT GGC CCT CAT GAA AAC AAG 3’
hCystatinB L73G DS:	5’ CTT GTT TTC ATG AGG GCC AGA TTG GAA CAC TCG 3’
Equistatind2 US:	5’CGG GAT CCA CCA CCA TGA AAG CCG CGT TAA CAC TTT G 3’
Equistatind2 DS:	5’TTT TCC TTT TGC GGC CGC GGC GGC CGC CTT GTC ATC G3’
DARPin-US:	5’ GGA TCC GAC CTG GGT AAG AAA CTG CTG 3’
DARPin-DS:	5’ TTT TCC TTT TGC GGC CTT GCA GG ATT TCA GCC AGG TC3’
$mBim_{EL}$ US:	5’ CGG GAT CCA CCA TGG CCA AGC AAC CTT CTG ATG 3’
$mBim_{EL}$ DS:	5’ TTT TCC TTT TGC GGC CGC TCA ATG CCT TCT CCA TAC CAG A3’
$mBim_{EL}$-ΔBH3-US:	5’ GGA TTG CAC AGG AGG AAA CTT ACA CAA GGA 3’
$mBim_{EL}$-ΔBH3-DS:	5’ TCC TTG TGT AAG TTT CCT CCT GTG CAA TCC 3’
$mBim_{EL}$S105/S109/T110A-US:	5’ CCG GCA CCC ATG GCA TGT GAC AAG GCA GCA CAA ACC 3’

mBim$_{EL}$S105/109/110A-DS: 5’ GGT TTG TGC TGC CTT GTC ACA TGC CAT GGG TGC CGG 3’

In the case of wild-type Bim$_{EL}$, it was also cloned into the BamHI/NotI sites of a lentiviral FG9-Loxp0mcherry-stop-Loxp inducible vector. The accuracy of all constructs was confirmed by sequencing.

2.3. Cell Culture and Transfection

MEFs were grown in DMEM supplemented with 10% FBS (Cat. No. F0926, Sigma-Aldrich, St. Louis, MO), and 2 mM L-glutamine (Cat. No. G3126, Sigma-Aldrich, St. Louis, MO). Cells were maintained at 37°C in humidified air containing 5% CO_2 and were routinely passaged every 2 days. Wild-type, Bim$^{-/-}$ and Bid$^{-/-}$ MEFs were kind gifts from Dr. David C. S. Huang (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). Wild-type and Bax$^{-/-}$/Bak$^{-/-}$ DKO MEFs were kind gifts from Dr. Craig B. Thompson (Memorial Sloan–Kettering Cancer Center, NY).

For generation of the stable cell lines expressing various cathepsin inhibitors, hCystatin B-Flag, Flag-Spi2a, and Equistatin d2-Flag were cloned into the FG9 lentiviral plasmid and cotransfected with pRRE, pHCMV and pRSV-Rev into HEK293T cells, using TransIT®-2020 according to manufacturer's instructions (Mirus®). Forty-eight hours later, viral supernatant was obtained and mixed (7 µg/ml) with polybrene (Hexadimethrine bromide, Cat. No. 107689,

Sigma-Aldrich, St. Louis, MO), filtered through a 0.45 μM filter (Millipore), and added to wild-type, $Bim^{-/-}$, and $Bax^{-/-}$/$Bak^{-/-}$ DKO MEFs respectively. The infected MEFs were subsequently expanded and selected with puromycin dihydrochloride (2 mg/ml) (Cat. No. 540411, Calbiochem, Billerica, MA) for 3 days, after which cathepsin inhibitor expression was detected by immunoblotting with an M2® Flag antibody.

For generation of CRISPR-Cas9 knockout cell lines, the following target sequence oligos were synthesized and digested with BsmBI (Esp3I) enzyme to create the same overhangs for annealing into lentiCRISPRv2 vector.

CRISPR-Cas9-mBim gRNA oligo-US:	5’ CAC CGG AAC CGC AAG GTA ATC CCG A 3’
CRISPR-Cas9-mBim gRNA oligo-DS:	5’ AAA CTC GGG ATT ACC TTG CGG TTC C 3’
CRISPR-Cas9-mCtL gRNA oligo-US:	5’ CAC CGT TCT GTG TGT GGA CTT CCA C 3’
CRISPR-Cas9-mCtL gRNA oligo-DS	5’ AAA CGT GGA AGT CCA CAC ACA GAA C 3’

2.4. Heat Shock Treatments

Cells were plated at 0.3−0.5×10^6 cells/well in 6-well plates 20 h prior to heat shock. Exposures were done in a tissue culture incubator at 44°C with 5% CO_2 for various periods of time, after which the cells were returned to a 37°C incubator for "recovery". Samples were collected for analyses at various time points post-heat shock.

2.5. Cell Death, Lysosomal Membrane Permeabilization (LMP), and Δψm assays

Trypsinized MEFs (3×10^5) were pelleted at 400 x g for 4 min, washed with PBS, and resuspended in 1 mL of Annexin V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Cells were then incubated with 100 ng/mL of Annexin-FITC for 5 min, and propidium iodide was added just prior to flow cytometric analysis. Recombinant Annexin V was expressed in BL21 (DE3)pLysS *E. Coli* and purified in-house on Ni^{2+}-NTA beeds by FPLC, labeled with FITC (1mg/mL) for 1.5 h, and dialyzed to remove unconjugated dye. Cell populations, labeled with FITC and/or PI, were analyzed by flow cytometry (BD Accuri C6®, BD, Franklin NJ). Similarly, to assess the loss in lysosomal membrane integrity and Δψm, either 100 nM of LysoTracker® Green DND-26 (Cat. No. L7526, ThermoFisher Scientific, Waltham MA) or 25 nM of tetramethylrhodamine (TMRE; Molecular Probes) were added into the sample well and incubated at 37°C for 20 min. Cells were then trypsinized and washed

one time with PBS and analyzed by flow cytometry.

2.6. Western Blotting

Cell pellets were lysed in RIPA lysis buffer (50mM Tris-HCl, pH7.4, 150mM NaCl, 1% v/v NP-40, 1% Na-deoxycholate, and 0.1% v/v SDS) on ice for 20 min, centrifuged at 18,000 x g for 15 min at 4°C, and the supernatants normalized for protein concentration by the Bradford assay. A 6x protein loading dye (0.5M Tris-HCl pH 6.8, 12g SDS, 47ml Glycerol, 9.3g DTT, 5% v/v β-mecaptoethanol, 60mg Bromophenol Blue) was then added to each samples, and equal amounts of protein were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and blocked in 0.1% Tween-TBS with 5% non-fat milk prior to incubation with primary antibodies (1:1,000–2,000). Appropriate secondary antibodies (1:3000-4000) were then used and blots were exposed to Perkin Elmer-Lighting Plus ECL (Cat. No. NEL104001EA Perkin Elmer, MA) and film (Cat. No. 30-810, Genesee Scientific, CA)

2.7. Cytochrome c release assay

Eight hours post-treatment, 2×10^6 cells were scraped, washed 1 X in PBS, and permeabilized in MOMP lysis buffer (20 mM HEPES, pH 7.4, 250 mM sucrose, 1 mM EDTA, 75 mM KCl, 2.5 mM $MgCl_2$) containing 0.05% digitonin (always added fresh) on ice for 5 min.

The cells were then centrifuged at 18,000 x g for 10 min at 4°C to collect the “cytosolic fractions”. The pellets were then lysed in RIPA buffer, as described above, to obtain the “mitochondrial fractions”. The protein concentrations of cytosolic and mitochondrial fractions were subsequently measured by the Bradford assay and resolved by SDS-PAGE.

2.8. Immunoprecipitation of cathepsin B and L

Cells were plated at $1x10^6$/plate in 100 mm tissue culture dishes 18-20 h prior to heat shock treatment. Six hours post-treatment, cells were scrapped and pelleted *via* centrifugation at 400 x g for 5 min, followed a wash with PBS. Cells were then lysed with a low pH lysis buffer (50 mM sodium acetate pH 6, 1mM EDTA, and 0.5% Triton X-100) on ice for 20 min. Finally, the cells were centrifuged at 18,000 x g for 10 min at 4°C to collect the supernatant, and the protein concentrations were measured and normalized by the Bradford assay. Anti-Flag® M2 MA Affinity Gel (Cat. No. A2220, Sigma-Aldrich, St. Louis, MO) were immediately added to each lysate and incubated overnight at 4°C. Protein bound beads were then pelleted *via* centrifugation at 14,000 x g for 2 min and washed with low the pH lysis buffer. Samples were then eluted into 2X non-reducing laemmli loading buffer (1M Tis-HCL pH6.8, 10% v/v SDS, 37.7g Glycerol, 60mg Bromophenol Blue), and the protein-protein interactions between cystatine B, cathepsin B, and cathepsin L were verified by western blotting with α-mCathepsin B (R&D system, AF965), α-h/mCathepsin L (R&D system,

MAB9521), or α-FLAG® M2 antibody (Sigma-Aldrich, F3165).

2.9. Measurement of Extracellular Cathepsin Activity

OmniCathepsin®-fluorogenic substrate, Z-FR-AMC (Cat. No. BML-P139-0010, Enzo Life Science), powder was dissolved in DMSO to a stock concentration of 50 mM. Cells were plated into 6-well plates ($3x10^5$/well) and treated with or without heat shock. Eight hours post-heat shock, 100 µL of sample media was incubated in a 96-well plate with Z-FR-AMC substrate (10 µM final concentration), and cathepsin activity was measured using a spectramax plate reader (Ex: 380 nm; Em: 460 nm). Cathepsin activity data was determined by measuring the initial velocity of the reaction.

2.10. Quantification of Confocal Immunofluorescence Images

Lysosome number was measured using ImageJ software with lysosomes approximated as circles. The images threshold was adjusted untill the smallest lysosome punctate appeared to be visible, and punctate lysosomes were selected with a size mask of 0 μm^2 to 1 μm^2. Thresholds were kept constant for all images. The colocalization between cathepsin B and lysosome was carried out using the colocalization plugin in ImageJ. Lysosomes were analyzed for total lysosome number and percentage of area in the cell and were individually plotted using Prism™ software, followed by a one-way ANOVA with a Tukey's post hoc analysis ($p<0.05$ was considered statistically significant). At lest eleven cells were

analyzed from each cell line.

2.11. Statistics

All experiments were performed at least three times, and each data point represents the mean ± S.E.M. Multiple group comparisons were performed by either one-way or two-way ANOVA, followed by a Tukey's post hoc analysis ($p<0.05$ was considered statistically significant).

Chapter 3 Bim mediates heat shock-induced cell death through Bax/Bak-dependent and -independent mitochondrial and lysosomal pathways.

3.1 Introduction

Heat shock is a type of ancient stress that has been explored for cancer therapy. However, the mechanisms that mediate heat shock-induced cell death are unclear, and heat shock alone is often insufficient to effectively kill cancer cells. Moreover, cancer cells often develop resistance to heat shock over time[94, 96, 122]. Therefore, heat shock has generally been combined with chemotherapeutic agents in order to improve treatment outcomes. A newer approach for the specific delivery of heat to tumors, including the use of nanoparticles, is also an area of active research. If we can reveal the signaling pathways that mediate heat shock-induced cell death, we can further understand and overcome some of the therapeutic resistance previously observed in cancer cells.

Cancer cells oftentimes undergo genetic and epigenetic modifications, resulting in, among other things, overexpression of anti-apoptotic BCL-2 family members and/or down-regulation of pro-apoptotic BCL-2 family members in order to escape the mitochondrial-dependent cell death pathway[123-126].

Therefore, we utilized specific genetic knockout mouse embryonic fibroblast (MEF) cell lines as tools to rule in or out BCL-2 family members in the heat shock response.

Our previous studies suggest that the canonical mitochondrial pathway is not essential for heat shock-induced cell death, nor are any of the established initiator caspases required for cell death[62, 63]. However, Green and colleagues have argued that the initiator caspase-2 is important for heat shock-induced cell death[59, 60]. In their model, caspase-2 mediates heat shock-induced cell death *via* cleavage of Bid, which in turn stimulates Bax/Bak oligomerization in the outer mitochondria membrane, leading to mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c. The released cytochrome c then stimulates formation of the Apaf-1-caspase-9 apoptosome complex, which activates the down stream effector caspase-3 and induces cell death[59, 60].

Furthermore, in our previous study, heat shock-induced caspase-3 activation could be inhibited by the poly caspase inhibitors, Z-VAD-FMK. Since heat shock does not appear to require initiator caspases, we speculate that another type of protease must be involved, and interestingly, Z-VAD-FMK has been shown to inhibit not only caspases but also some lysosomal cysteine proteases [127]. Thus, we hypothesized that lysosomal proteases, particularly

cysteine proteases, might act as the initiator protease(s) in heat shock-induced cell death.

Some studies have suggested that lysosomal-mediated cell death requires the BH3-only family member Bid in order to engage the mitochondrial pathway and amplify the cell death signal[6, 57, 121]. Our study indicates that Bid is not essential for lysosomal membrane permeabilization (LMP) following heat shock. We found instead that another BH3-only family member, Bim, plays a critical role in mediating lysosomal-dependent cell death in response to heat shock.

Thus, the actual mechanisms responsible for heat shock-induced cell death remain controversial and unclear. In this chapter, we have utilized genetically-defined knockout MEFs to evaluate whether the mitochondrial and/or lysosomal signaling is essential for heat shock-induced cell death.

3.2 Results

3.2.1. Caspase-2 and Bid are not essential for heat shock-induced cell death, but may serve to amplify the cell death signal at longer exposures to heat shock.

To revisit the potential role of the caspase-2→Bid→Bax/Bak→MOMP→apoptosome pathway that proposed by Green and colleagues, we first examined caspase-$2^{-/-}$ MEFs for their resistance to heat shock-induced cell death. In Figure 3.1 (panel A), loss of caspase-2 partially blocked cell death at a lower dose of heat shock (44°C 1h), but failed to suppress cell death at a higher dose (44°C 1.5h). Similarly, when we examined cells for a loss in mitochondrial membrane potential ($\Delta\psi m$) through staining with Tetramethylrhodamine Ethyl Ester (TMRE) followed by flow cytometry (Figure 3.1, panel B). Caspase-$2^{-/-}$ MEF was partially resistant to a loss $\Delta\psi m$ at the lower dose of heat shock, but there was no significant difference between wild-type and caspase-$2^{-/-}$ MEFs at the higher dose of heat shock. Interestingly however, despite failing to block heat shock-induced cell death particularly at the higher dose of heat shock, the caspase-$2^{-/-}$ MEFs did exhibit less caspase-3 activation at both doses (Figure 3.1 panel C). These results suggest that caspase-2 is not essential for heat shock-induced cell death, but that it did play a role in regulating the activation of caspase-3.

Previously Grütter and colleagues engineered an ankyrin repeated protein (DARPin) that selectively inhibits caspase-2[128]. This DARPin inhibits caspase-2 in *vitro* with a subnanomolar inhibition constant, in contrast to other more non-specific peptidic caspase-2 inhibitors such as Ac-VDVAD-CHO and Ac-LDESD-CHO[128]. Therefore, we synthesized and cloned DARPin into a lentiviral expression system and stably expressed it in both wild-type and $Bax^{-/-}/Bak^{-/-}$ DKO MEFs. In response to heat shock treatment, DARPin-expressing cells behaved like caspase-$2^{-/-}$ MEFs that it partial protected cells from heat shock-induced cell death at a lower dose, but not at a higher dose of heat shock (Figure 3.1 panel D). Similar to caspase-$2^{-/-}$ cells, DARPin also blocked caspase-3 activation in response to heat shock treatment (Figure 3.1 panel E). This result provided first evidence that the artificial caspase-2 inhibitor DARPin could inhibit caspase-2, not only in *vitro*, but also in *vivo*.

Since Bid can be cleaved by caspase-2, we next examined Bid knockout MEFs and once again discovered that loss of Bid only partially protected cells from heat shock (Figure 3.1, panel F). Collectively, these findings suggest that caspase-2 and Bid do not play an essential role in heat shock-induced cell death, but may serve to amplify the cell death signal, particularly at lower dose of heat shock.

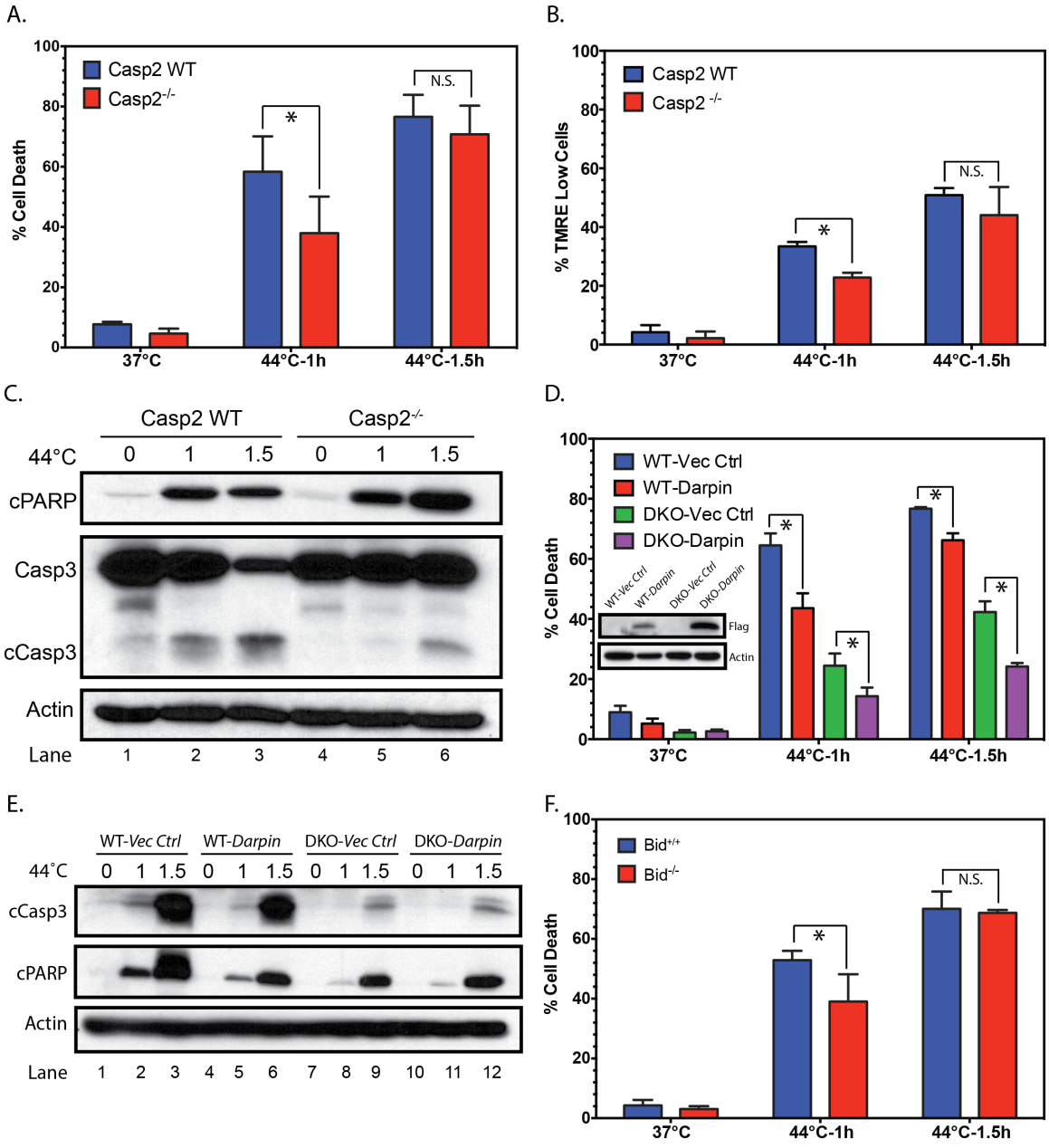

Figure 3.1. Caspase-2 and Bid participate in, but are not essential for heat shock-induced cell death.

Wild-type, caspase-$2^{-/-}$, and Darpin expressing MEFs were exposed to heat shock (44°C for 1-1.5 h) in a humidified incubator (5% CO_2 - 95% air). The cells were then transferred to a 37°C incubator and later collected for caspase-3 activation, PARP cleavage (24 h, *panels C and E*), Δψm (24 h, *panel B*), and cell death measurements (24 h, *panels A and D*). Similarly, Wild-type, and $Bid^{-/-}$ MEFs were exposed to same treatment and later collected for cell death measurements (24 h, *panel F*). (*: $P<0.05$; N.S.: No significant difference)

3.2.2. Bax and Bak are partially involved in heat shock-induced cell death.

Our data suggested that Caspase-2 and Bid were not essential for heat shock-induced cell death, which raised doubts about the previously proposed caspase-2→Bid→Bax/Bak→MOMP→apoptosome pathway. Bax and Bak are essential of MOMP; therefore $Bax^{-/-}/Bak^{-/-}$ DKO MEFs are highly resistant to most kinds of stressful stimuli, including DNA damaging agents, ER stressors, growth factors withdrawal, *etc*.[129-131]. In contrast, even though the BH3-only protein Bid was not essential for cell death, we presumed that $Bax^{-/-}/Bak^{-/-}$ DKO MEFs would be highly resistant. To our surprise, however, while loss of Bax and Bak completely blocked MOMP, ~50 % of treated cells died in response to heat shock (Figure 3.2, panel A). By contrast, $Bax^{-/-}/Bak^{-/-}$ DKO MEFs were completely resistant to UV irradiation treatment (Figure 3.2, panel D and E). More intriguingly, $Bax^{-/-}/Bak^{-/-}$ DKO MEFs still underwent PARP cleavage and caspase-3 activation, followed by heat shock (Figure 3.2, panel C). Therefore, these data suggest that heat shock induces cell death through both Bax/Bak-dependent and -independent signaling pathways.

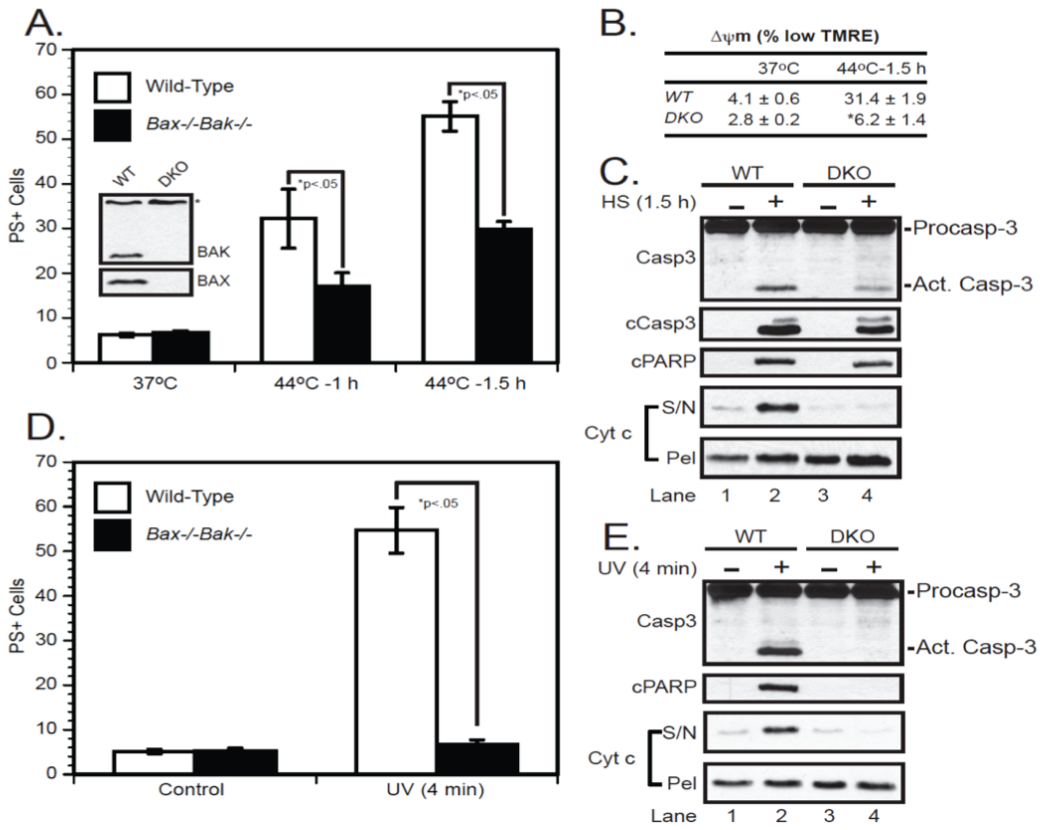

Figure 3.2. Bax$^{-/-}$/Bak$^{-/-}$ DKO cells resist heat shock-induced MOMP and Δψm, but still undergo caspase-3 activation and cell death.
Wild-type and Bax$^{-/-}$/Bak$^{-/-}$ DKO cells were exposed to (A-C) heat shock (44°C for 1-1.5 h) in a humidified incubator (5% CO_2- 95% air), or (D and E) UV irradiation (4 min) on a transilluminator. The cells were then transferred to a 37°C incubator and later collected for MOMP (16 h), caspase-3 activation, PARP cleavage (24 h, *panels C and E*), Δψm (24 h, *panel B*), and cell death measurements (24 h, *panels A and D*). cPARP, cleaved PARP; cCasp3, cleaved/active caspase-3; S/N, supernatant; Pel, mitochondrial pellet; *p<0.05, significantly different from wild-type cells.

(Mahajan and Chen *et al*., 2013)

3.2.3. The Apaf-1-Caspase-9 apoptosome is not essential for heat shock-induced cell death.

Based upon the previous data, we knew that heat shock induced cell death through both Bax/Bak-dependent and -independent pathways. Therefore, it was important to determine whether the apoptosome played an important role in the Bax/Bak-dependent pathway following MOMP. In order to answer this question, we first generated a caspase-9 dominant-negative construct by mutating its active site cysteine (C287A). Casp9 (C287A) acts as a dominant negative by competing with endogenous caspase-9 for recruitment to oligomerized Apaf-1 following cytochrome c release, and that blocks caspase-9 activation.

We therefore expressed of casp9 (C287A) in wild-type cells and assessed its ability to inhibit heat shock-induced cell death. As before, casp9 (C287A) partially inhibited cell death at the lower dose of heat shock, but it had no significant effect compared to the vector control at the higher dose (Figure 3.3, panel A). Interestingly, casp9 (C287A) also protected cells from loss of TMRE staining at the lower dose, but not the higher dose (Figure 3.3, panel B). This suggested that, like caspase-2 and Bid, caspase-9 played a role in amplifying the cell death signal at the lower dose. It is worth noticing that caspase-9 can reportedly feedback directly on mitochondria[132-134], and following its activation of caspase-3, caspase-9 can also cleave Bid.

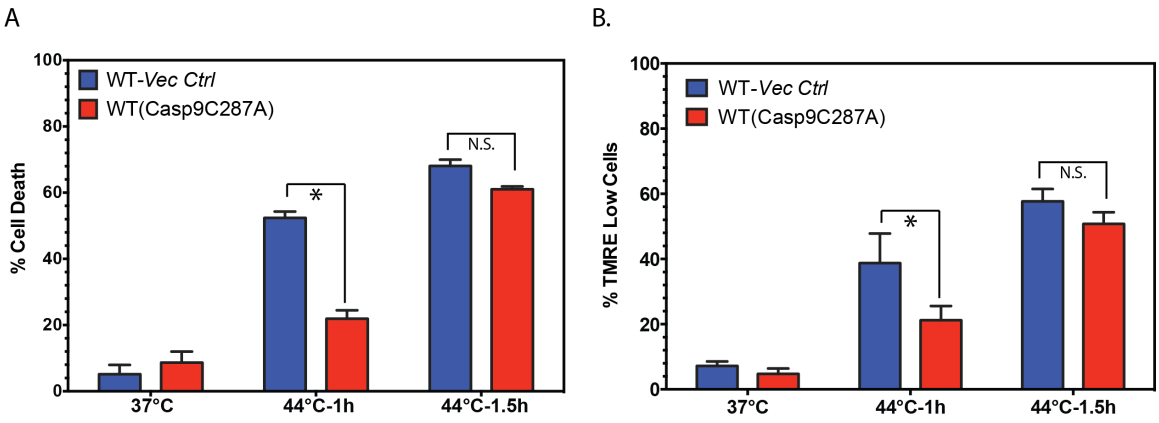

Figure 3.3. Caspase-9 is not essential for heat shock-induced cell death. Vector control MEFs and those expressing caspase-9 (C287A) were exposed to heat shock (44°C for 1–1.5 h) in a humidified incubator (5% CO2–95% air). The cells were then transferred to a 37°C incubator and later collected for cell death and Δψm measurements (24 h, *panel A and B*). (*: $P<0.05$; N.S.: No significant difference)

3.2.4 BH3-only family member Bim is essential in heat shock-induced cell death.

To this point in our studies, loss of caspase-2, Bid, and caspse-9 failed to dramatically suppress heat shock-induced cell death. Particularly following lower dose of heat shock exposure. Moreover, while loss of Bax and Bak also failed to fully inhibit cell death, $Bax^{-/-}/Bak^{-/-}$ DKO cells were only more resistant than $Bid^{-/-}$ cells. Therefore, this raised the question as to another BH3-only family member might contribute in heat shock-induced cell death signaling. We therefore compared heat shock-induced cell death in $Bim^{-/-}$ MEFs as well as in $Bid^{-/-}$ MEFs. To our astonishment, unlike in $Bid^{-/-}$ MEFs, $Bim^{-/-}$ MEFs were completely resistant to heat shock-induced cell death, even more so than in $Bax^{-/-}/Bak^{-/-}$ DKO MEFs (Figure 3.4, panel A). Loss of Bim completely inhibited heat shock-induced MOMP, cytochrome c release, caspase-3 activation and PARP cleavage (Figure 3.4, panel B). These results suggested that Bim was playing a crucial role in heat shock-induced Bax/Bak-dependent, but also Bax/Bak-independent cell death.

To be certain that our results were not due to a clonal artifact, we reintroduced mouse Bim (mBim) back into the $Bim^{-/-}$ MEFs and verified that reintroduction of mBim re-sensitized $Bim^{-/-}$ MEFs to heat shock-induced cell death. Since Bim is a potent pro-apoptotic BH3-only family member, we were unable to use our standard lentiviral system as Bim expression killed the viral packaging cells. In order to overcome this difficulty, we inserted a Loxp-mcherry-

stop codon-Loxp (LSL) cassette upstream of the mBim coding sequence in our lentiviral construct (Figure 3.5, panel A). This allowed us to generate LSL-mBim viruses and infect $Bim^{-/-}$ MEFs. These cells were then infected with a tamoxifen-inducible Cre-ER recombinase. Upon addition of tamoxifen, Cre-ER translocates of inducible Bim gene into the nucleus, recombines the Loxp fragment, thereby removing mcherry-stop codon and inducing expression of mBim. Using this system, we reintroduced $Bim^{-/-}$ MEFs with mBim ($Bim^{-/-}$;mBim), and performed heat shock. As expected, reintroduction of Bim restored cell death in $Bim^{-/-}$ MEFs to a level virtually identical to that of the vector control cells (Figure 3.5, panel B). Reintroduction of Bim also resensitized $Bim^{-/-}$ MEFs to heat shock-induced MOMP, caspase-3 activation, and PARP cleavage (Figure 3.5, panel C and D). Finally, while the $Bim^{-/-}$ MEFs were more resistant to heat shock than the $Bax^{-/-}$/$Bak^{-/-}$ DKO MEFs, we were unsure if this represented a true biological effect or was due to an artifact.

If $Bim^{-/-}$ cells are more resistant than $Bax^{-/-}$/$Bak^{-/-}$ DKO cells, it would indicates that Bim acted upstream of not only the Bax/Bak-dependent but also the Bax/Bak-independent pathways. To address this question, we utilized the CRISPR-Cas9 genome editing technology to knock out the endogenous Bim allele in both wild-type and $Bax^{-/-}$/$Bak^{-/-}$ DKO MEFs. As expected, knockout of Bim in wild-type MEFs had dramatically reduced cell death in response to heat shock (Figure 3.5, panel E). More excitingly, however, deletion of Bim from $Bax^{-/-}$

/Bak$^{-/-}$ DKO cells rendered these cells completely resistant to heat shock-induced cell death (Figure 3.5, panel E). Thus, this suggested that Bim is acting as an upstream player in mediating both heat shock-induced Bax/Bak-dependent and -independentcell death.

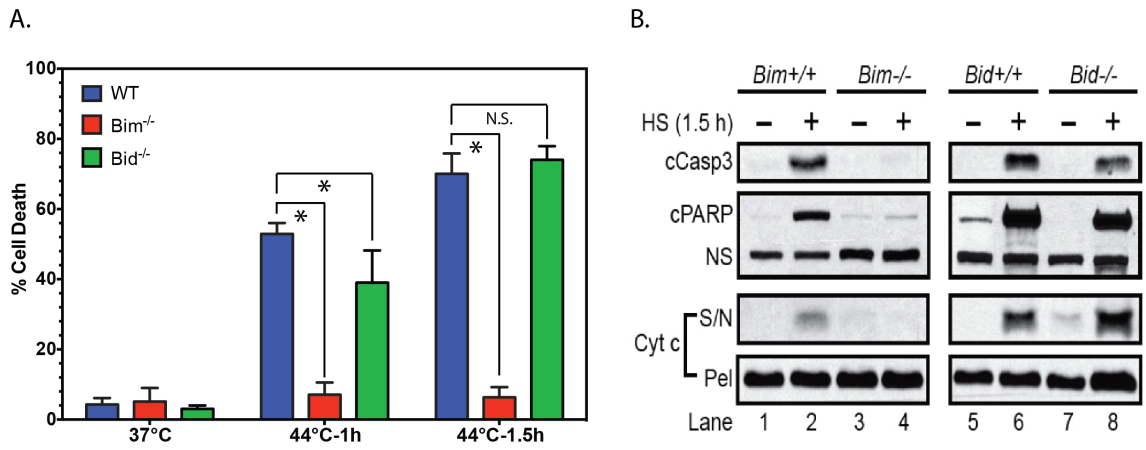

Figure 3.4. Bim is critical for heat shock-induced cell death.
Wild-type, $Bim^{-/-}$, and $Bid^{-/-}$ MEFs were exposed to heat shock (44°C for 1–1.5 h) in a humidified incubator (5% CO_2–95% air). The cells were then transferred to a 37°C incubator and later collected for MOMP (16 h), caspase-3 activation, PARP cleavage (24 h, *panel B)*, and cell death measurements (24 h, *panel A*). *p<0.05, significantly different from wild-type cells. (*: *P*<0.05; N.S.: No significant difference)

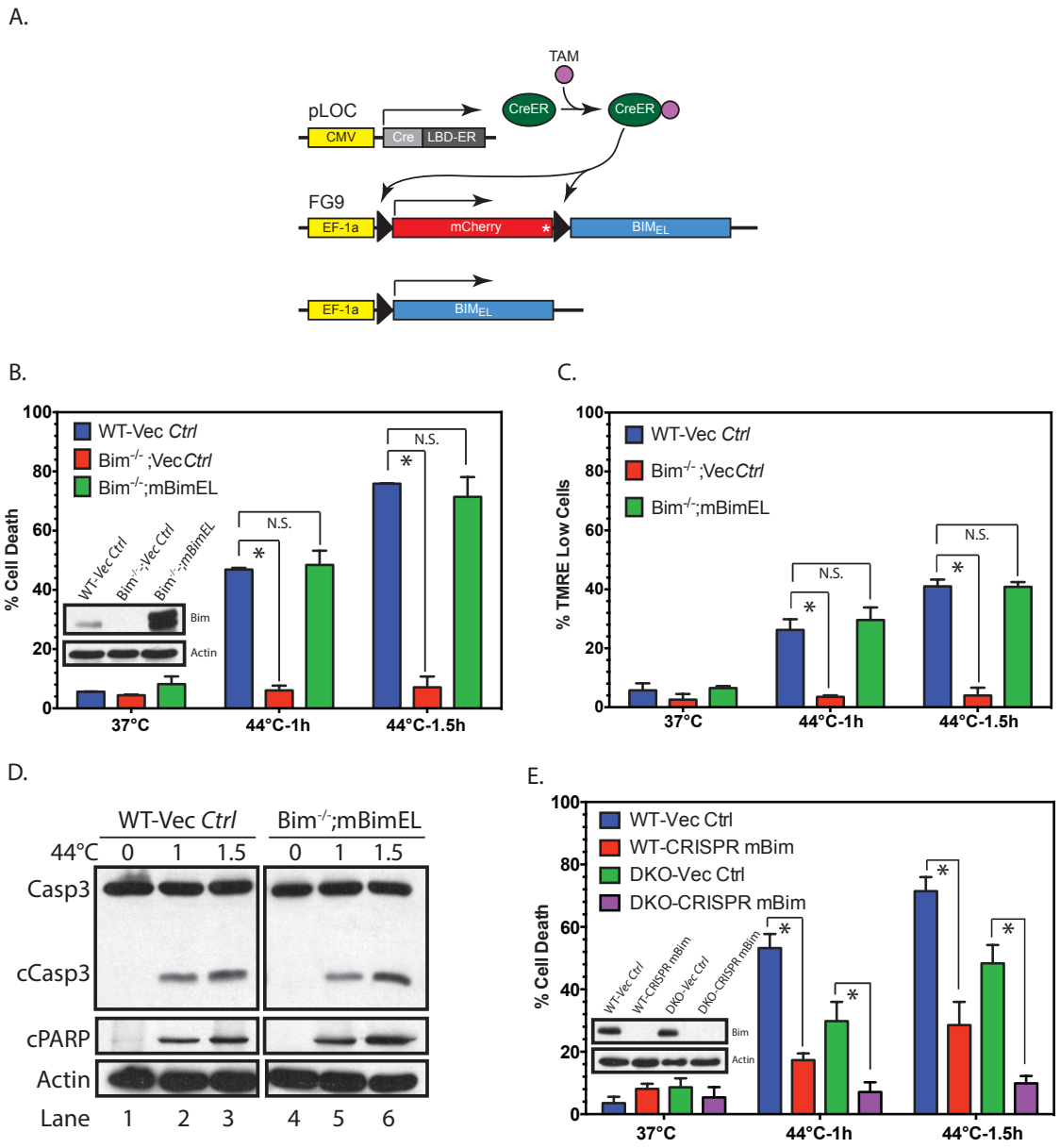

Figure 3.5. Bim is essential for heat shock-induced cell death.
Wild-type and Bim$^{-/-}$ MEFs expressing either the vector control or the LSL-mBim cassette (CA) were exposed to tamoxifin and then heat shocked (44°C for 1–1.5 h) in a humidified incubator (5% CO_2–95% air). The cells were subsequently transferred to a 37°C incubator and later collected for caspase-3 activation, PARP cleavage (24 h, *panel C)*, Δψm (24 h, *panel B*), and cell death measurements (24 h, *panel A*). Similarly, CRISPR-Cas9 generated vector control, Bim$^{-/-}$, and Bax$^{-/-}$/Bak$^{-/-}$/Bim$^{-/-}$ MEFs were exposed to the same heat shock treatment, and cells were assayed for cell death (24h, *panel D*). (*: P<0.05; N.S.: No significant difference)

3.2.5 Heat shock induces lysosomal membrane permeabilization in a Bim-dependent but Bax/Bak-independent manner.

Bim plays an essential role in mediating heat shock-induced cell death through both Bax/Bak-dependent and -independent pathways. While initiator caspases-2 and -9 participate in the former pathway at lower dose of treatment, neither is essential for regulating heat shock-induced cell death. However, our previous studies suggested that Z-VAD-FMK, a broad-spectrum caspase inhibitor, could effectively block the downstream activation of caspase-3 and suppress heat shock-induced cell death[62]. Therefore, we speculate that another protease(s) must be activated or released following heat shock. Though Z-VAD-FMK is a well-known established caspase inhibitor, it can also inhibit papain-like cysteine cathepsins, including cathepsin B, L, V, K, F, S, X, H, and C [127]. Therefore, we hypothesized that a lysosomal cysteine cathepsin might be acting as the apical protease in heat shock-induced cell death.

Previous evidence indicates the specific stressful stimuli can induce lysosomal membrane permeabilization (LMP), resulting in the release of lysosomal proteases (cathepsins) into the cytoplasm. The cathepsins can then cleave Bid (tBid), which in turn activates Bax and Bak in order to engage the mitochondrial cell death pathway[5, 6, 11, 12, 57, 58]. In order to assess whether heat shock induced LMP, we first performed immunofluorescence staining with antibodies to cathepsin B and stained nucleus with DAPI in control wild-type cells

and those exposed to heat shock. To untreated control cells, cathepsin B inside within lysosomes, as indicated by a well-distributed punctate pattern of staining throughout the entire cytoplasm. However, in response to heat shock, lysosomal membrane rupture resulted in the release of cathepsin B into the cytoplasm and a more diffuse staining pattern (Figure 3.6). These data suggested that heat shock induces LMP and cathepsin release into the cytosol.

We examined wild-type, $Bim^{-/-}$, $Bid^{-/-}$, and $Bax^{-/-}/Bak^{-/-}$ DKO MEFs for evidence of LMP following heat shock by staining cells with LysoTracker Green and analyzed them by flow cytometry. Notably we found that $Bax^{-/-}/Bak^{-/-}$ DKO and $Bid^{-/-}$ cells underwent LMP following heat shock treatment; whereas, $Bim^{-/-}$ MEFs remained entirely resistant (Figure 3.7, panel A and B). Reintroduction of Bim into $Bim^{-/-}$ cells resensitized them to heat shock-induced LMP (Figure 3.7, panel E). We also simultaneously measured heat shock-induced LMP and MOMP $\Delta\Psi m$, at numerous time points following heat shock and found that LMP began to occur in some wild-type cells almost immediately, whereas MOMP was not observed until 6-8h post treatment (Figure 3.7, C and D), interestingly that LMP proceeded MOMP by several hours following heat shock. More remarkably, while $Bim^{-/-}$ MEFs were almost entirely resistant to LMP and MOMP over the entire time course, $Bax^{-/-}/Bak^{-/-}$ DKO MEFs showed only a delay in LMP, even though they remained resistant to MOMP. $Bid^{-/-}$ MEFs behaved essentially the same as wild-type cells.

Taken together, despite previous reports that lysosomal-mediated cell death is Bid and Bax/Bak dependent manner[2, 4-7, 57, 121], our results indicate that heat shock induces lysosomal-mediated cell death in a Bim-dependent, but largely Bax/Bak-independent manner. Moreover, heat shock-induced LMP was an early event and suggested that cathepsins are acting as apical proteases in this lysosomal cell death signal.

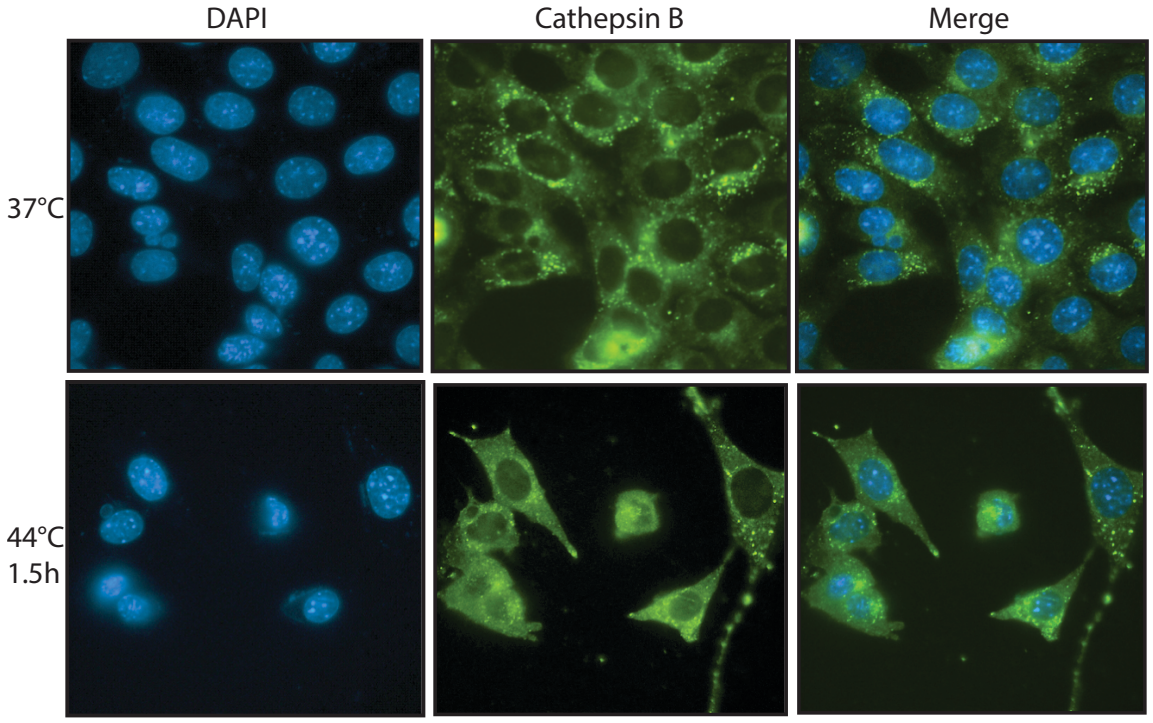

Figure 3.6. Heat shock induces LMP and the release of cathepsin B into the cytosol.
Cells were seeded onto coverslips 22 h prior to treatment, exposed to heat shock (44°C for 1.5 h) in a humidified incubator (5% CO_2- 95% air), transferred to a 37°C incubator, and subsequently fix in 4% formaldehyde. Immunofluoresce staining was performed with anti-cathepsin B antibody and the nucleus was counter stained with DAPI.

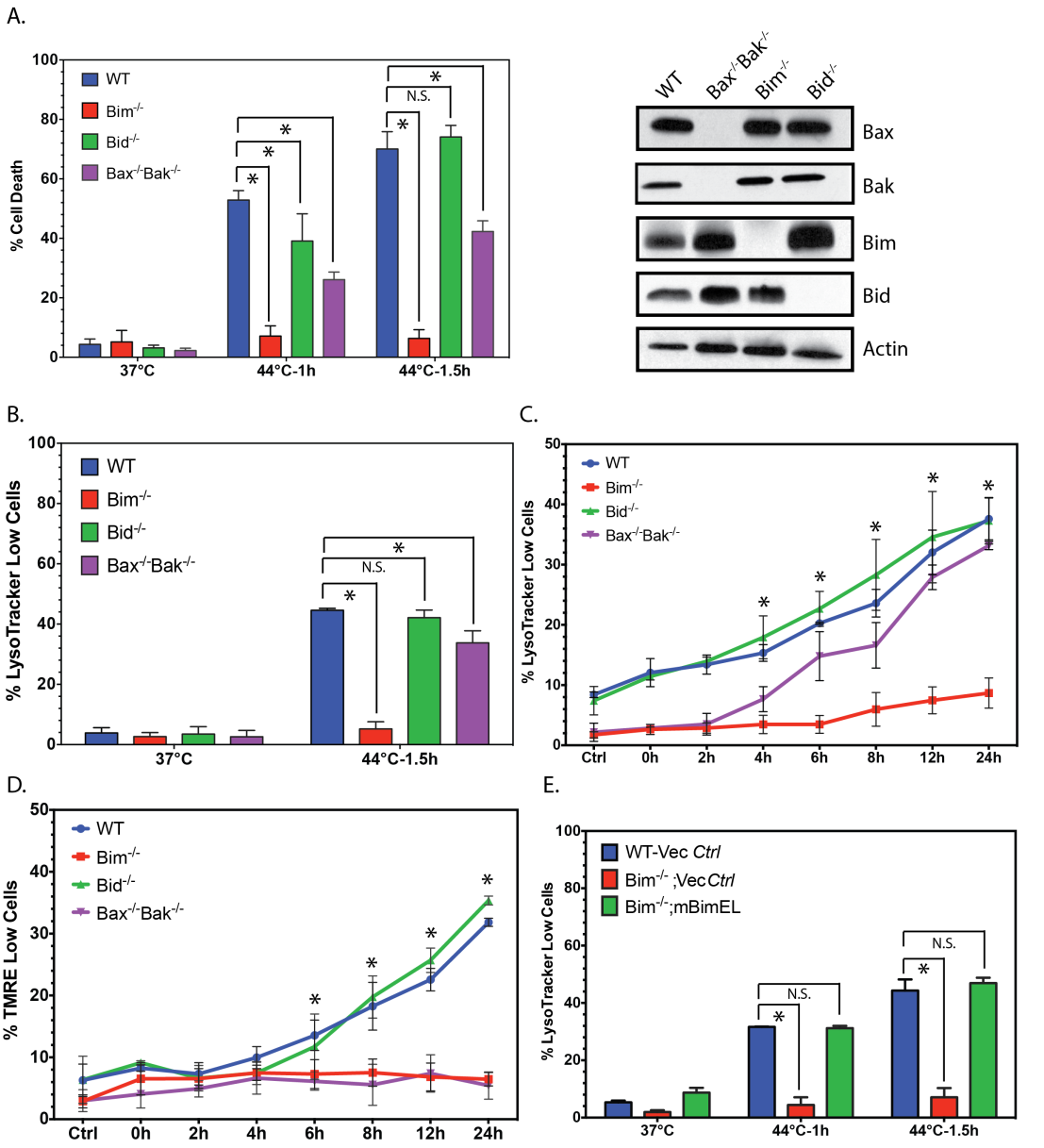

Figure 3.7. Heat shock induced lysosomal cell death independent of Bax/Bak, yet dependent on Bim.
Wild-type, $Bax^{-/-}/Bak^{-/-}$ DKO, $Bim^{-/-}$, and $Bid^{-/-}$ cells were exposed to heat shock (44°C for 1–1.5 h) in a humidified incubator (5% CO_2–95% air). The cells were then transferred to a 37°C incubator and later collected for lysosomal membrane permeabilization (LMP) and cell death measurements (24 h, *panels A and B*). Heat shock-induced LMP and Δψm were also measured immediately after the treatment throughout 24h post-heat shock (*panels C and D*). Similarly, vector control and mBim reintroduced cell cells were collected for heat shock-induced LMP (24h, *panel E*). (*: $P<0.05$; N.S.: No significant difference)

3.2.6 Overexpression of cystatin B suppresses heat shock-induced cell death.

To this point, our data had shown that heat shock induces LMP and release of cathepsins into cytoplasm. However, we had not directly demonstrated that cathepsins were responsible for mediating cell death. There are three classes of cathepsins: serine, cysteine, and aspartic proteases based on their catalytic residues[1, 2, 4, 5, 9, 11, 12, 16, 135]. Overall, there are more than thirteen different cathepsins that could potentially participate in heat shock-induced cell death. Therefore, in order to narrow down the list of cathepsins that contribute to heat shock-induced cell death, we stably expressed in cells cathepsin inhibitors that target each class of cathepsins. We expressed serine cathepsin inhibitor, Spi2a [136], cysteine cathepsin inhibitor, Stefin B/cystatin B (CSTB) [135, 137-140], and the aspartic acid cathepsin D inhibitor, Equistatin d2, which is derived from the sea anemone (actinia equina) [141]. Cells expressing of each of these individual cathepsin inhibitors were exposed to heat shock and cell death was measured using AnnexinV/Pi staining and flow cytometry.

Surprisingly, we found that only CSTB effectively suppressed heat shock-induced cell death and that inhibition of cathepsin D with Equistatind2 actually enhanced cell death (Figure 3.8, panel A). More surprisingly, CSTB not only inhibited MOMP (as indicated by TMRE staining) but also suppressed LMP, indicating that, once released, cysteine cathepsins appeared to participate in the

LMP of other lysosomes within the cell, or alternatively, promoted the death of neighboring cells. The effect of CSTB was specific as a binding mutant failed to suppress LMP or MOMP (Figure 3.8, panel B and C).

Finally, we also stably expressed CSTB or its binding mutant into both wild-type and $Bax^{-/-}/Bak^{-/-}$ DKO MEFs. Cells expressing CSTB were resistant to heat shock-induced caspse-3 activation, PARP cleavage, and cell death. In wild-type expressing CSTB cells, cytochrome c was also suppressed especially at the lower dose of heat shock (Figure 3.9, panel A and B). As before, the CSTB binding mutant failed to suppress cell death (Figure 3.9, panel A). To confirm that CSTB bound and inhibited cysteine cathepsins, we performed immunoprecipitations using M2 Flag antibodies to pull down CSTB. As expected, CSTB interacted with both cathepsin B and cathepsin L in response to heat shock (Figure 3.9, panel C). Thus, our results suggested that cystatin B inhibits heat shock-induced cell death through inhibition of the cysteine cathepsins.

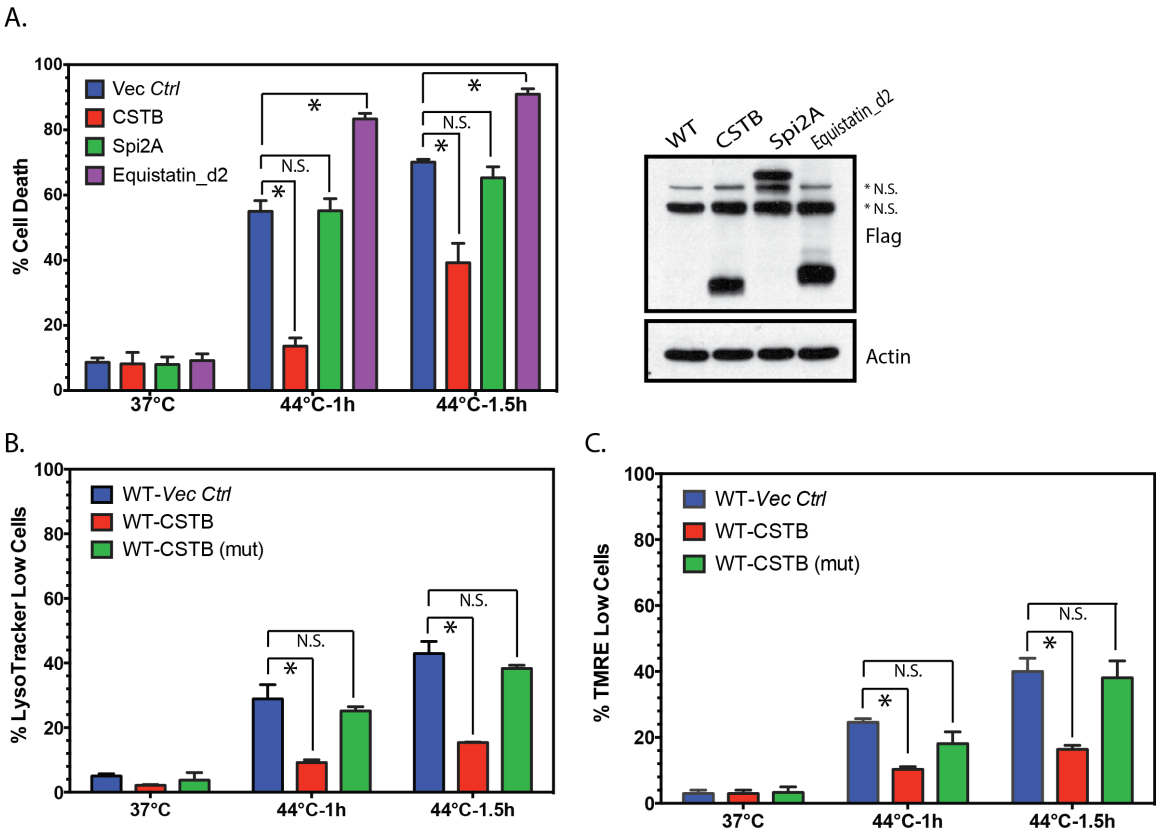

Figure 3.8. Cysteine cathepsins are crucial for heat shock-induced cell death.

Cells stably-expressing the cathepsin inhibitors, CSTB, Equistatind2, or Spi2A were exposed to heat shock (44°C for 1-1.5 h) in a humidified incubator (5% CO_2-95% air). The cells were then transferred to a 37°C incubator and later collected for LMP, Δψm, and/or cell death measurements (24 h, *panels A, B, and C*). (*: *P*<0.05; N.S.: No significant difference) (*N.S.: non-specific signal)

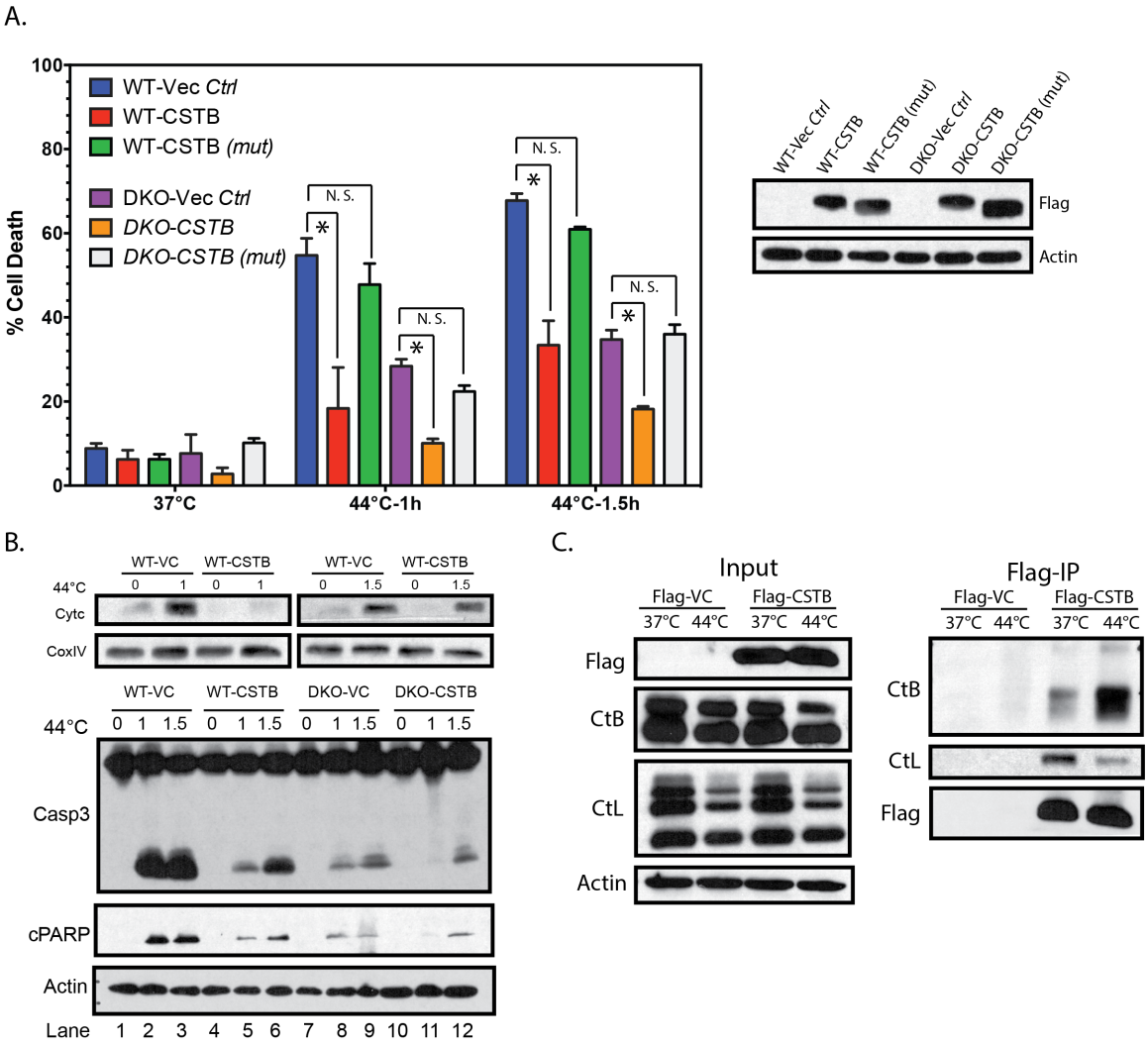

Figure 3.9. CSTB binds to cysteine cathepsins and attenuates heat shock-induced caspase-3 activation and cell death.
Cells stably-expressing CSTB or its binding mutant were exposed to heat shock (44°C for 1-1.5 h) in a humidified incubator (5% CO_2-95% air). The cells were then transferred to a 37°C incubator and later collected for measurements of cell death, caspase-3 activation, PARP cleavage, and cytochrome c release (24 h, *panels A and B*). The interaction of CSTB with cysteine cathepsin B and L was detected by immunoprecipitation (*panel C*). (*: P<0.05; N.S.: No significant difference)

3.2.7. Cathepsin L is essential for heat shock-induced cell death.

Based on our data with CSTB, cysteine cathepsins were clearly playing an important role in heat shock-induced cell death. However, it was unclear if specific cathepsins played a predominant role in cell death. CSTB bound to both cathepsin B and L in response to heat shock. Therefore, we obtained and performed heat shock experiments in cathepsin B ($CtB^{-/-}$) and cathepsin L ($CtL^{-/-}$)-deficient cells. Interestingly, while loss of cathepsin B did not significantly protect cells from heat shock, compared to wild-type cells (Figure 3.10, panel A), $CtL^{-/-}$ cells were completely resistant to heat shock-induced LMP and cell death (Figure 3.10, panel A and B). Moreover, reintroduction of mouse cathepsin L into $CtL^{-/-}$ cells ($CtL^{-/-}$;mCtL) restored heat shock-induced LMP and cell death (Figure 3.10, panel A and B). The fact that knockout of the cathepsin L gene provided somewhat better protection from heat shock, compared with wild-type cells overexpressing CSTB, is probably due to incomplete inhibition of CtL, particularly following the higher dose of heat shock.

Bim regulates heat shock-induced cell death through both Bax/Bax-dependent and -independent pathways, so that loss of Bax and Bak provided only approximately 50% protection from cell death. By contrast, loss of cathepsin L led to resistance that mirrored of the Bim-deficient cells, implying that cathepsin L participated in both Bax/Bak-dependent and -independentkilled. To confirm this assumption, we knocked out endogenous cathepsin L gene *via* CRISPR-Cas9

technology in wild-type and $Bax^{-/-}/Bak^{-/-}$ DKO cells. As predicted, loss of cathepsin L dramatically suppressed LMP and cell death in both cases (Figure 3.10, panel C and D). This suggested that cathepsin L appears to function upstream of Bax/Bak in heat shock-induced cell death, but can kill independent of Bax/Bak, MOMP, and apoptosome formation. While it seemed most probable that Bim acted upstream of cathepsin L, this could not yet be firmly established since both loss of Bim and cathepsin L suppressed LMP, MOMP, and cell death to a similar extent.

To summarize the findings of this chapter (Figure 3.11), Bim initiates heat shock-induced cell death signals *via* both Bax/Bak-dependent and -independent pathways. While Bim may directly engage Bax and Bak to induce MOMP, Bim-induced LMP appears to play a more prominent role in cell death. This Bim dependent lysosomal cell death pathway begins with lysosomal membrane permeabilization and cathepsins release. Release of cathepsin L in particular appears to be critical and it apparently participates in its own release perhaps by cleaving critical lysosomal membrane proteins following heat shock. Indeed, overexpression of CSTB or deletion of cathepsin L (but not cathepsin B) suppressed LMP and cell death. Notably, cathepsins are capable of cleaving Bid[4-6, 57, 106] and tBid may participate in MOMP, thereby amplifying the cell death signal following heat shock.

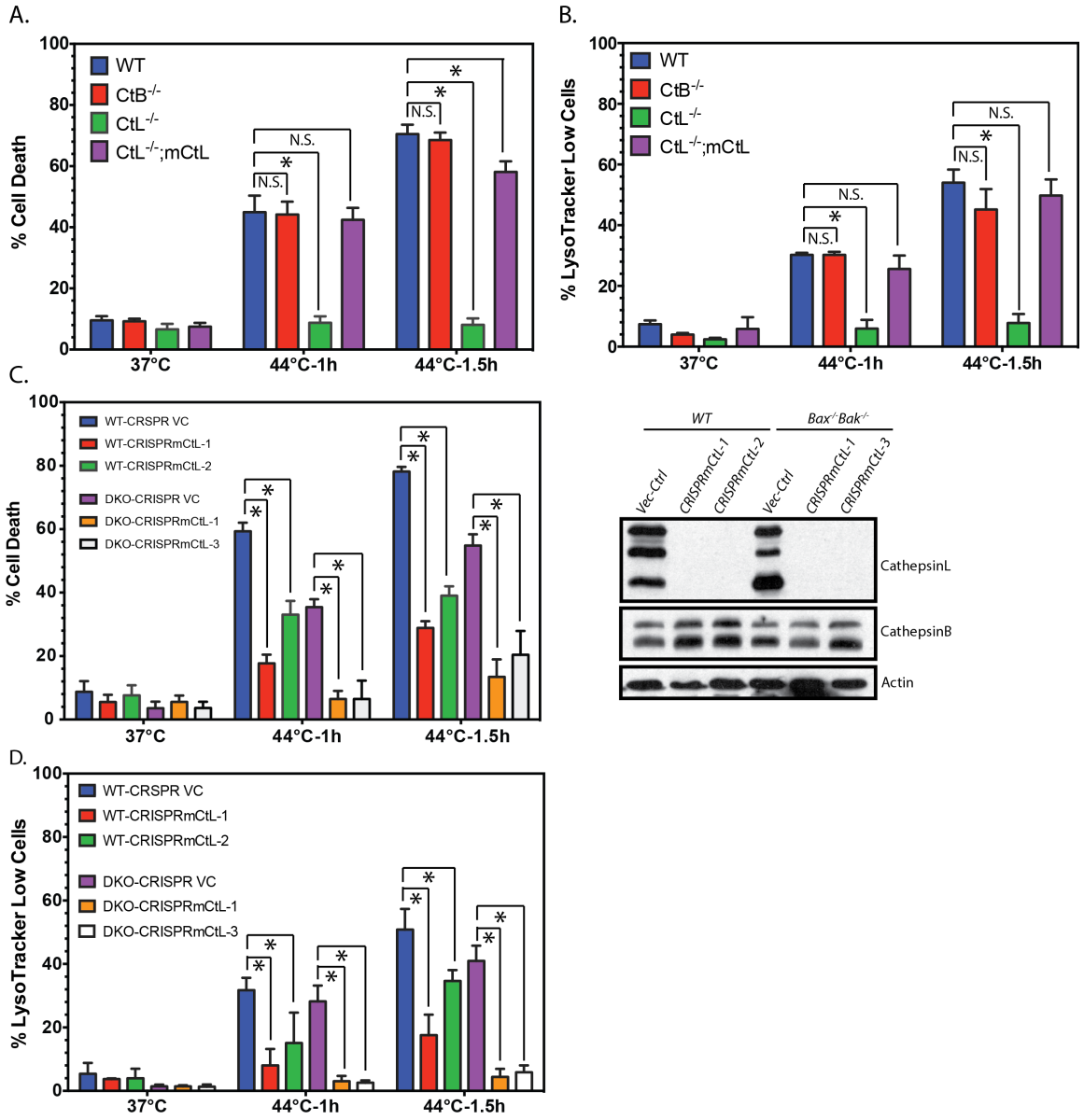

Figure 3.10 Cathepsin L is responsible for heat shock-induced cell death. Wild-type, CtB$^{-/-}$, CtL$^{-/-}$ and CtL$^{-/-}$;mCtL cells were exposed to heat shock (44°C for 1–1.5 h) in a humidified incubator (5% CO_2–95% air). The cells were then transferred to a 37°C incubator and later collected for LMP and cell death measurements (24 h, *panels A and B*). Similarly, CRISPR vector control and CtL$^{-/-}$ cells were collected for the measurement of heat shock-induced LMP and cell death (24h, *panel C and D*). (*: P<0.05; N.S.: No significant difference)

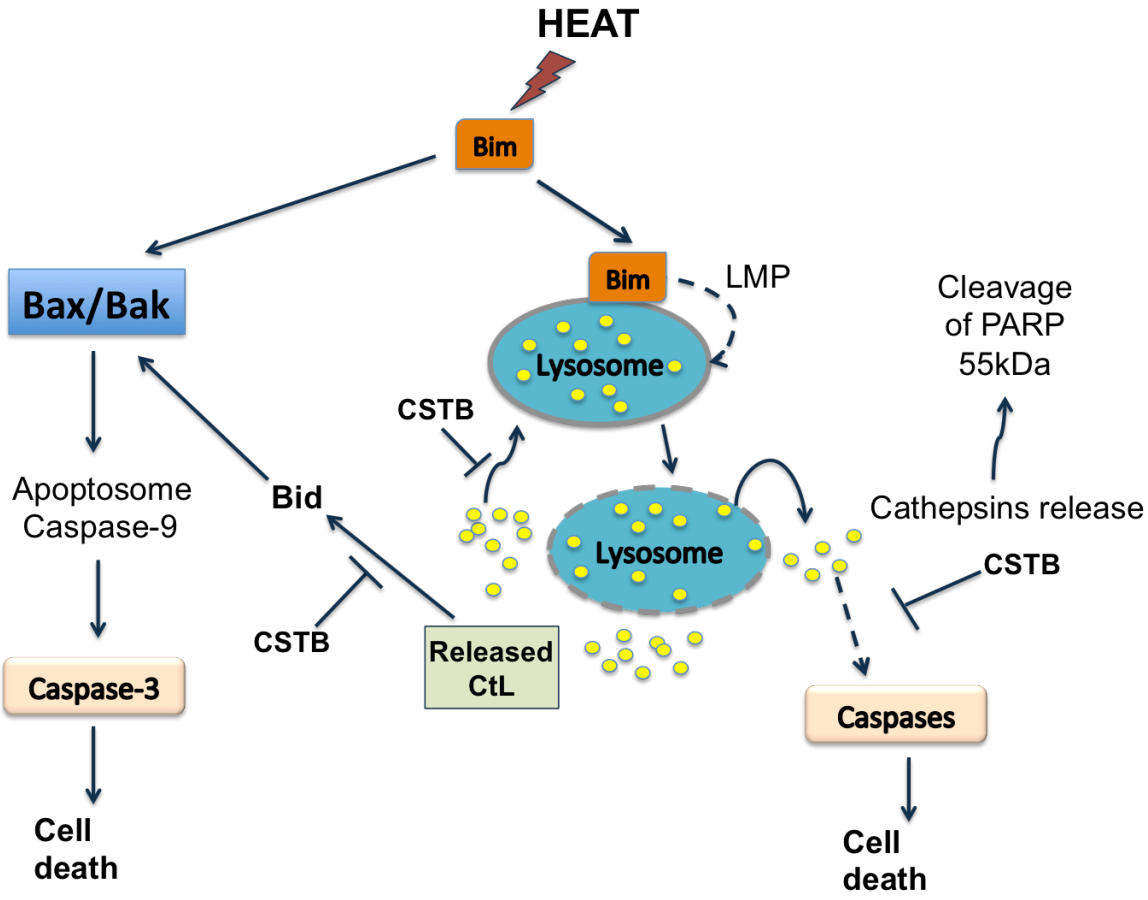

Figure 3.11. Model of heat shock-induced cell death.
In response to heat shock, the BH3-only protein Bim initiates the apoptotic signal *via* both Bax/Bak-dependent and Bax/Bak-independent pathways. Bim can induce MOMP by activating Bax and / or Bak, resulting in cytochrome c release, apoptosome formation and caspase activation. However, Bim also mediates LMP and the release of cathepsins independent of Bax and Bak. Once released, cathepsins may cleave Bid and participate in MOMP or directly cleave other cellular substrates, perhaps including caspases.

3.3 Discussion

Previously, we have shown that initiator caspases are not essential for heat shock-induced cell death; however, Z-VAD-FMK, a broad-spectrum caspase-inhibitor, provided significant protection from cell death[62]. Others have instead suggested that caspase-2 and Bid are essential for heat shock-induced cell death[59, 60]. Therefore, controversy has surrounded the mechanism by which heat shock kills cells. In this chapter, we have demonstrated that heat shock induces cell death *via* Bax/Bak-dependent and – independent pathways, and that Bim but not Bid is primarily responsible for initiating the cell death signaling in response to heat shock.

$Bax^{-/-}/Bak^{-/-}$ DKO cells are resistant to many types of stimuli, such as DNA damage, ER stressor, *etc*. Therefore, it is perhaps unsurprising that Bim could mediate cell death *via* Bax and Bak following heat shock. However, it is remarkable that loss of Bax and Bak provides only ~50% of protection from heat shock, while loss of Bim provides virtually complete protection. Moreover, our observation that heat shock induces LMP, upstream of MOMP, and does so in a Bim-dependent, but Bid or Bax/Bak-independent manner, is entirely novel and striking.

DNA damaging agents, Fas ligand, and interleukin-3 (IL-3) deprivation all reportedly induce LMP in a Bax/Bak dependent manner, amplifying cell death downstream of or parallel to MOMP[5, 16, 58, 142]. It has been also suggested that so-called lysosomal-mediated cell death is not a single signaling event that relies entirely on the lysosomal proteases. In another words, once released, cathepsins may cleave the BH3-only family member Bid, which in turn activates Bax/Bak to engage the mitochondrial cell death pathway[3-5, 9, 57, 106, 121, 139, 143]. Still other studies suggest that some cathepsins, such as cathepsin B, and L can directly cleave caspase-3, and cathepsin D can cleave caspase-8[11, 12, 105, 106, 139, 144-146].

Our data, however, suggest that Bid does not play a critical role in heat shock-induced cell death, whereas cathepsin L is essential. Interestingly, cathepsin L is one of the most highly secreted and ubiquitously expressed cathepsins, and it has the ability to digest extracellular matrix and initiate anoikis[147]. Another cysteine cathepsins, cathepsin G is important in regulating focal adhesion kinase and regulates anoikis in cardiomyocytes[31, 148-150]. In our hands, deficiency of cathepsin L restored cells almost entirely resistant to heat shock-induced LMP and cell death. Moreover, in preliminary data we have found that addition of the recombinant CSTB to the media of heat-shocked cells suppresses cell death and facilitates long-term survival (data not shown). Similarly, $Bim^{-/-}$ cells also continue to proliferate 72h post-heat shock. Thus we speculate that secreted cathepsins as well as intracellular cysteine cathepsins

following LMP play a role in regulating cell death. Precisely, how Bim mediates LMP remains unclear, since it contains no transmembrane domain. In Chapter 4, we will provide further insight into its probable roles in LMP.

Jäättelä and colleagues have reported that heat shock protein 70 (HSP70) promotes cell survival by inhibiting LMP following certain stressful stimuli[151]. They find that HSP70 stabilizes lysosomes by binding to the endo-lysosomal lipid bis(monoacylglycero)phosphate (BMP), an essential co-factor for lysosomal sphingomyelin metabolism. The HSP70–BMP interaction specifically inhibits the activity of acid sphingomyelinase, an important enzyme that hydrolyzes sphingomyelin, thereby preventing lysosomal injuries[98, 152-155]. Despite HSP70, excessive heat stress still induces sphingomyelinase activity and ceramide generation. Moreover, increased ceramide and heat shock have reported to activate JNK phosphorylation[40, 52, 156-161]. The presence of ceramide may result in a more fragile lysosomal membrane that is more prone to heat shock-induced LMP. Interestingly, it has reported HSP70 can effectively inhibit JNK/Bim activation pathway in UV-induced cell death[156] and inhibits heat-induced cell death by preventing Bax translocation to mitochondria and MOMP[162]. Therefore, we speculate that HSP70 may paly a role in $Bim^{-/-}$ cells to inhibit heat shock-induced LMP. We could knockout the HSP70 using CRISP-Cas9 technology in $Bim^{-/-}$ cells to determine if it resensitizes $Bim^{-/-}$ cells to heat shock-induced LMP and cell death. If loss of HSP70 in this context restored cell death from heat

shock, it would suggest that HSP70 plays a role in stabilizing lysosomal membrane following heat shock.

Our data indicate that cysteine cathepsins- and in particular cathepsin L- are important for heat shock-induced cell death. We hypothesize that both intracellular cathepsins and secreted cathepsins are important in response to heat shock-induced cell death. In order to address this hypothesis, we could overexpress cystatin C, which is normally secreted in both wild-type and $Bax^{-/-}$ /$Bak^{-/-}$ DKO cells. Cystatin C inhibits secreted cathepsins and regulates cathepsin L during p53-induced cell death[135, 137, 138, 163, 164]. Since CSTB inhibits heat shock-indcued LMP and cell death by interacting with cysteine cathepsins (Figure 3.8, panel B and C; Figure 3.9, panel C), cystatin C may also inhibit heat shock-induced cell death if extracellular cathepsin L participates in cell death signaling.

Notably, while CSTB suppressed heat shock-induced cell death, overexpression of Equistatind2 actually enhanced cell death (Figure 3.8, panel A) Interestingly, cathepsin D has been shown to cleave Apaf-1 binding protein (AVEN), thereby disrupting assembly of apoptosome[165]. This may explain why inhibition of cathepsins D with Equistatind2 sensitized cells to heat shock. In addition, cathepsin D reportedly degrades endogenous CSTB, thereby attenuating its inhibition to cysteine cathepsins[145]. Thus, cathepsin D may play a role in regulating AVEN and cystatin B in response to heat shock. We could

potentially knockout or overexpress cathepsin D in wild-type and $Bax^{-/-}/Bak^{-/-}$ DKO to confirm our results with Equistadind2 and evaluate the effects of cathepsins D on AVEN and CSTB cleavage following heat shock. In doing so, we could have a clearer idea as to the role of cathepsin D plays in heat shock-induced cell death.

Chapter 4. Bim-mediated lysosomal membrane permeabilization following heat shock.

4.1 Introduction

In Chapter 3 we demonstrated an essential role for Bim-induced cell death, with Bim mediating both Bax/Bak-dependent and -independent cell death pathways. Bim triggered MOMP and release of cytochrome c through activation of Bax/Bak. Once released, cytochrome c stimulated Apaf-1 assembles into the apoptosome complex, resulting in the recruitment and activation of caspase-9 and its downstream substrate, the effector caspase-3. However, Bim simultaneously mediated lysosomal cell death following heat shock and independent of Bax and Bak. LMP and the resulting cytosolic cathepsins may have contributed to Bax/Bak activation and MOMP by cleaving Bid, and likely cleaving membranes and additional substrates. However, the release of cathepsins into the extracellular space also appeared to contribute to cell death.

Despite the characterization of these pathways; however, it remained unclear how Bim mediated LMP in response to heat shock. Bim is thought to be sequestered by its interaction with LC8 in the dynein motor complex[28, 52, 53, 55], and this complex competes with kinesin to regulate the mobility of organelles such as lysosome and Golgi along microtubules[81, 82]. Thus they are important in

organelle trafficking and the sorting of various protein in the cell[81]. Since Bim interacts with the dynein motor complex, we speculate that Bim might regulate the lysosomal and/or cathepsin trafficking prior to or in response to heat shock.

4.2. Results

4.2.1 Bim regulates heat shock-induced LMP and cell death independent of its BH3 domain.

Our findings in Chapter 3 revealed that Bim played an essential role in mediating heat shock-induced LMP and cell death independent of Bax/Bak. The underlying mechanisms by which Bim achieved these results however remained unknown. Since Bim is a BH3-only protein, we began by assessing whether its BH3 domain was essential for killing. We first introduced a key point mutation (G154E)[28] and generated a deletion mutant that completely lacked of its BH3 domain (BimΔBH3).

Bim (G154E) and Bim (ΔBH3) were individually expressed in $Bim^{-/-}$ MEFs and heat shocked as before. To our great surprise, both Bim (G154E) and Bim (ΔBH3) resensitized $Bim^{-/-}$ cells to heat shock-induced LMP and cell death, similar to wild-type cells. However, $Bim^{-/-}$ cells expressing the vector control remained fully resistant to heat shock (Figure 4.1, panel A and B). Thus, disruption of Bim's interaction with Bax/Bak as well as anti-apoptotic BCL-2 family members was not required for killing.

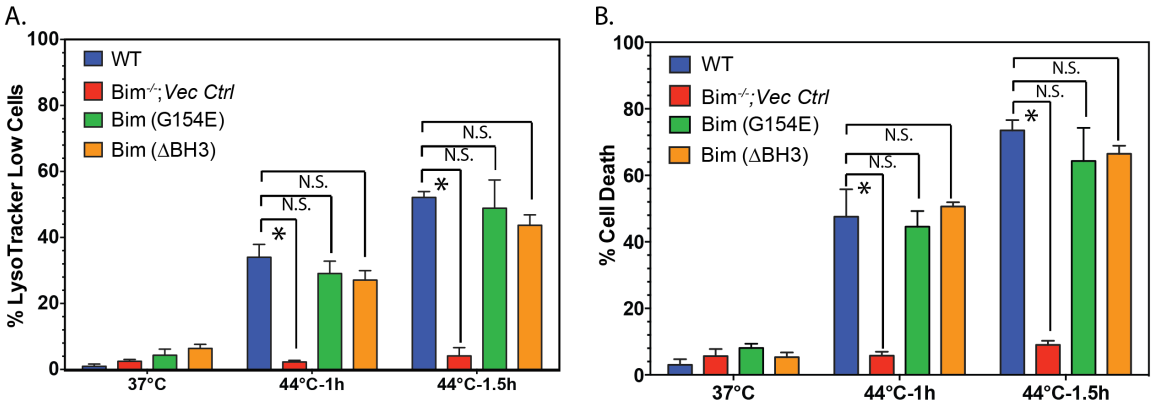

Figure 4.1. Bim mediates heat shock-induced LMP and cell death independent of its BH3 domain.

Vector control cells and those stably expressing of Bim (G154E) and Bim (ΔBH3) were exposed to heat shock (44°C for 1-1.5 h) in a humidified incubator (5% CO_2- 95% air). The cells were then transferred to a 37°C incubator and later collected for measurements of LMP and cell death (24 h, *panels A and B*). Both BH3 mutants of Bim restored heat shock-induced LMP and cell death. (*: $P<0.05$; N.S.: No significant difference)

4.2.2 Loss of Bim does not suppress heat shock-induced cell death by inducing autophagy.

BH3 domains were originally thought to be important in regulating apoptosis through their binding to other BCL-2 family member proteins. However, Beclin-1 also possesses a BH3 domain, and with both VPS34 and UVRAG, forms a complex that regulate autophagy[166-168]. But its BH3 domain is not required for this activity.

Notably, by binding to Beclin-1, BCL-2 disrupts formation of the Beclin-1/VPS34/UVRAG complex and suppresses autophagy. Conversely, BH3-only proapoptotic members can bind to BCL-2, displace Beclin-1, and induce Beclin-1/VPS34/UVRAG complex formation, resulting in increased autophagy[166]. Interestingly, as already noted, Bim can bind to the microtubule-associated dynein motor complex under the normal physiological conditions through its interaction with LC8[28, 50]. Following certain types of cellular stress, the interaction between LC8 and the dynein motor complex is disrupted which unleashes Bim and allows it to translocate together with LC8 to the outer membrane of mitochondria, where it can bind to neutralize anti-apoptotic BCL-2 family members such as BCL-2, and/or activate Bax and Bak[28, 50, 52, 55].

Intriguingly, Rubinztein and colleagues have recently suggested that Bim interacts with Beclin-1. This interaction does not require Bim's BH3 domain, but

is facilitated by LC8. Loss of Bim or disruption of this interaction results in the translocation of Beclin-1 from microtubules to ER, thereby promoting autophagosome formation and autophagy[52, 53, 55, 166]. Therefore, we questioned if loss of Bim suppressed heat shock-induced cell death by upregulating basal autophagy. To answer this question, we compared heat shock-induced cell death in both ATG5$^{-/-}$ and ATG16L1$^{-/-}$ MEFs (Figure 4.2), as both ATG5 and ATG16L1 are essential proteins in autophagy. Interestingly, we found that neither loss of ATG5 nor ATG16L1 sensitized cells to heat shock when compared with wild-type cells (Figure 4.2, panel A). This result suggested that loss of Bim does not protect cells from heat shock by inducing autophagy.

To further confirm this result, we generated mutants of Bim in which serine 105, serine 109, and threonine 110 residues were mutated to alanine (S105/109A/T110A, or STA). These mutations prevent Bim from interacting with LC8[28, 53]. We then reconstituted Bim$^{-/-}$ MEFs with wild-type, Bim (G154E), and Bim (G154E/STA) then assessed basal autophagy by western blotting for LC3 lipidation (LCII), and SQSTM1/p62 turnover.

Interestingly, following heat shock an accumulation of LCII was detected in both wild-type and Bim (G154E) cells, but not Bim (G154E/STA) cells (Figure 4.2, panel B). However, autophagic turnover of p62 was reduced in wild-type and Bim (G154E) cells, compared to Bim (G154E/STA) cells (Figure 4.2, panel B).

This result suggests that the association of Bim with LC8 led to heat shock-induced LMP, and that loss of functional lysosomes prevented autophagic turnover of p62, even though upstream autophagic signals and LC3 lipidation remained intact. In short, heat shock appeared to prevent autophagy by permeabilizing the lysosomes necessary to carry it out.

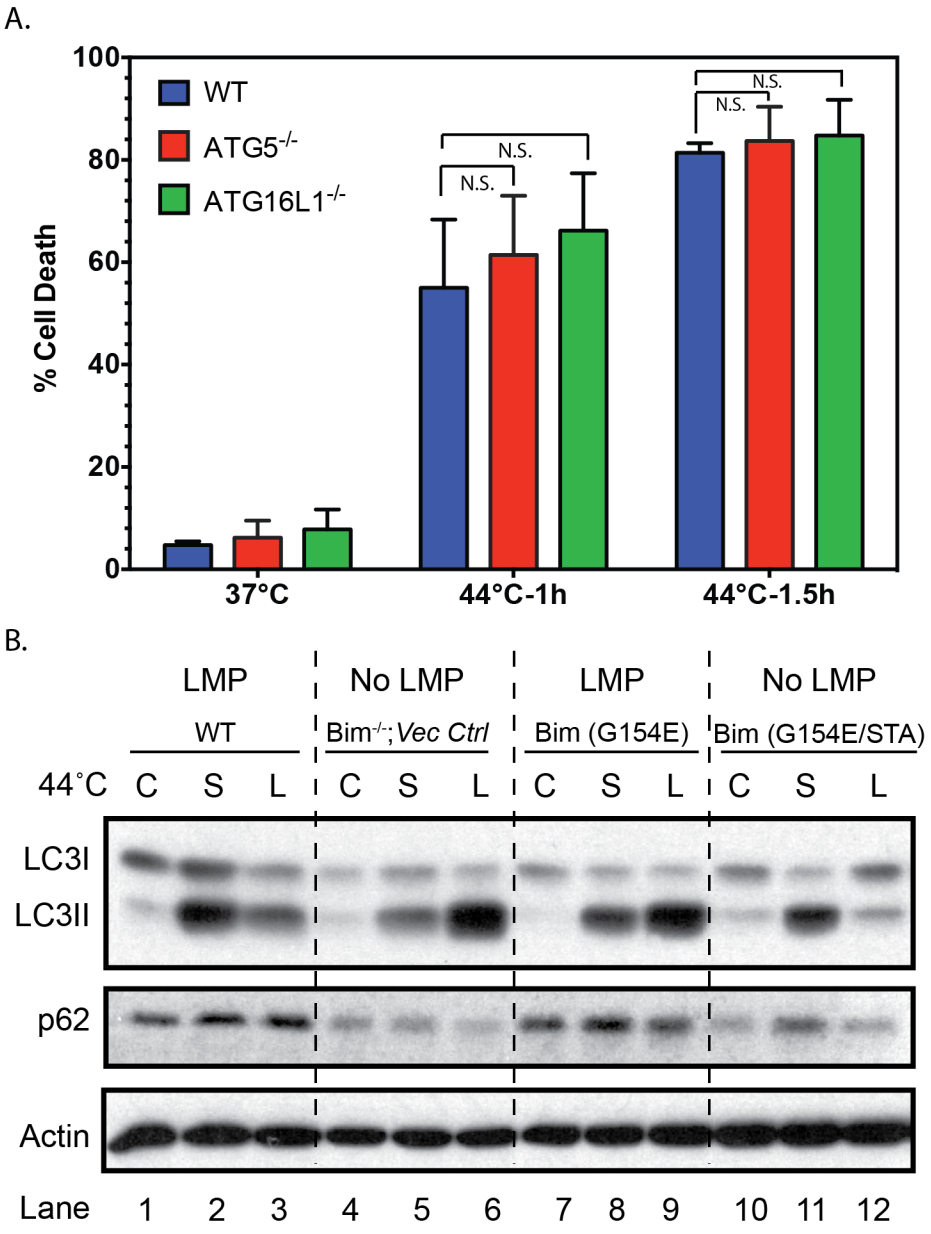

Figure 4.2. Autophagy is not responsible for the resistance of Bim$^{-/-}$ to heat shock-induced cell death.
Wild-type, ATG5$^{-/-}$ and ATG16L1$^{-/-}$ MEFs were exposed to heat shock (44°C for 1-1.5 h) in a humidified incubator (5% CO_2- 95% air). The cells were then transferred to a 37°C incubator and later collected for measurement of cell death (24 h, *panel A*). Likewise, vector control, Bim$^{-/-}$ cells, and cells stably expressing Bim (G154E) and the LC8 binding mutant, Bim (G154E/STA), were collected and assayed for autophagy markers LC3, and SQSTM1/p62 (24h *panel B*). (*: P<0.05; N.S.: No significant difference; C: control; S: 44°C-1h; L: 44°C-1.5h)

4.2.3 Bim's interaction with LC8 of the dynein motor complex is essential for heat shock-induced LMP and cell death.

Loss of Bim mediates resistance to heat shock and an increase in autophagy. However, autophagy did not appear to be responsible for the resistance of $Bim^{-/-}$ cells to heat, as loss of neither ATG5 nor ATG16L1 enhanced cell death. Nevertheless, Bim is sequestered to microtubules *via* its binding with LC8 of the dynein motor complex. Therefore, we speculated this interaction might play an important role in the response to heat shock, over and done with simple sequestration of Bim. Since the Bim (G154E/STA) mutant failed to interact with LC8, we compared $Bim^{-/-}$ cells reconstituted with this mutant or Bim (G154E). Heat shock-induced LMP and cell death were analyzed in these cell lines using and AnneinV/Pi staining, respectively (Figure 4.3).

Remarkably reintroduction of Bim (G154E/STA) failed to restore heat shock-induced LMP or cell death, exhibiting completely resistance similar to $Bim^{-/-}$ cells (Figure 3.4, panel A). Bim (G154E), on the other hand, resensitized $Bim^{-/-}$ cells to heat shock-induced cell death (Figure 4.3, panel A). Unlike wild-type and Bim (G154E)-expressing cells, neither $Bim^{-/-}$ nor Bim (G154E/STA)-expressing cells underwent heat shock-induced LMP, PARP cleavage, or activation of caspase-3 (Figure 4.3, panel B and C). To summarize, the interaction between Bim and dynein motor complex-LC8 was essential for heat shock-induced LMP and cell death.

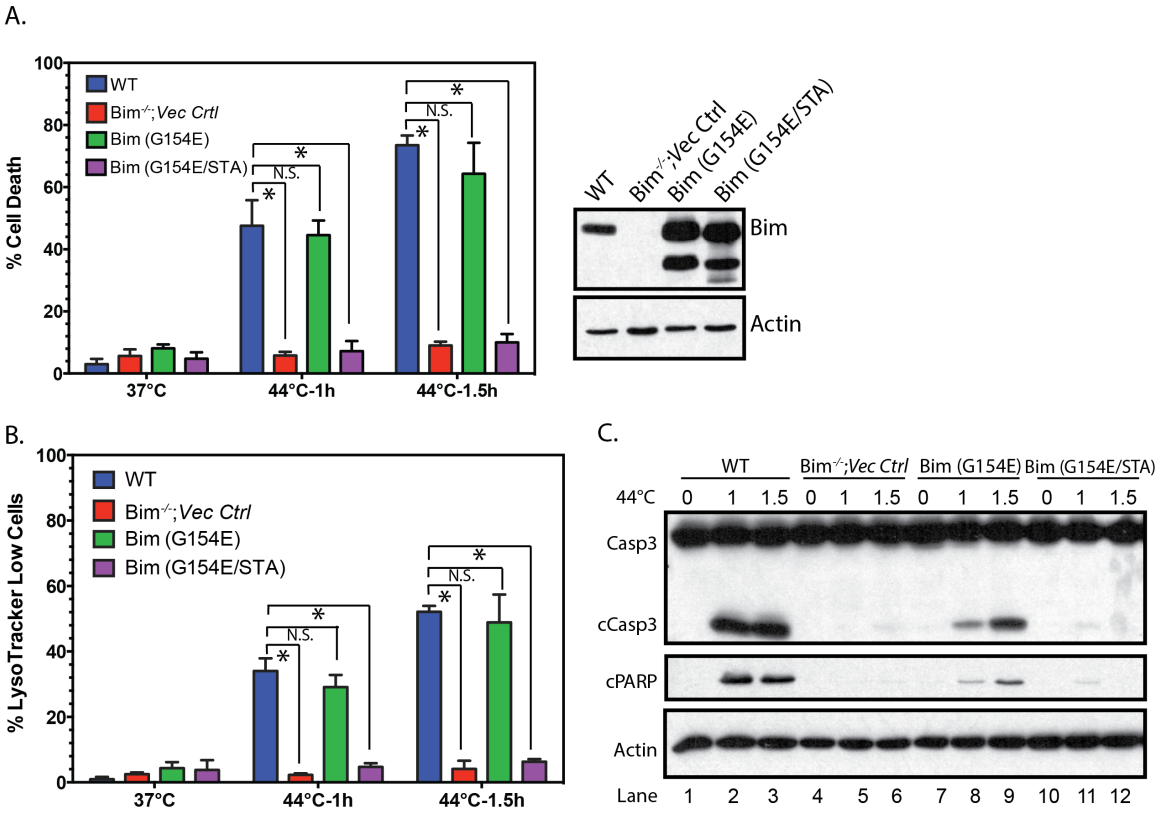

Figure 4.3. Bim mediates heat shock-induced LMP and cell death through its interaction with LC8.

Cells expressing vector control, Bim (G154E), or the LC8 binding mutant Bim (G154E/STA) were exposed to heat shock (44°C for 1-1.5 h) in a humidified incubator (5% CO_2- 95% air). The cells were then transferred to a 37°C incubator and later collected for LMP and cell death measurements (24h *panel A and B*). Likewise, cells were collected and immunoblotted for caspase-3 activation and PARP cleavage (24h *panel C*).

(*: *P*<0.05; N.S.: No significant difference)

4.2.4. Bim's interaction with LC8 impacts lysosome number, position, and secretion of cathepsins.

Bim mediates heat shock-induced LMP and cell death through its interaction with LC8 of the dynein motor complex. The dynein motor complex along with Kinesin regulates the trafficking of organelles, in which lysosomes, and impact protein sorting[81, 83]. Lysosome-mediated cell death is usually initiated by the release of cathepsins following LMP, but normal secretion of cathepsins into the extracellular space also plays important roles in cell attachment and migration. Since abnormal detachment of cells can lead to cell death (anoikis), we sought to determine if Bim might mediate cell death through both LMP and increased secretion/activation of extracellular cathepsins.

We first examined culture medium for extracellular cathepsin activity in *vitro* using Ominicathepsin®-AMC (Z-FR-AMC) fluorogenic substrate, which can be cleaved by most cysteine cathepsins (Figure 4.4). We found that medium from both $Bim^{-/-}$ and Bim (G154E/STA)-expressing cells possessed considerably lower extracellular cathepsin activity, compared with wild-type, Bim (G154E), and Bim (ΔBH3)-expressing cells (Figure 4.4). Thus, Bim appeared to regulate the secretion of cathepsins *via* its interaction with LC8 and independent of its BH3 domain.

Next, we examined the lysosomes in each cell line *via* immunofluorescence staining with cathepsin B and LAMP-1, in order to determine if Bim affected lysosomal number or position under normal physiological conditions (Figure 4.5, panel A). After analysis of the confocal microscopic images using ImageJ software, we observed dramatic difference in both the number of lysosomes and their subcellular distribution in $Bim^{-/-}$ and Bim (G154E/STA)-expressing cells compared with wild-type cells and those expressing Bim (G154E) or Bim (ΔBH3). Unlike wild-type, Bim (G154E), and Bim(ΔBH3)-expressing cells, both $Bim^{-/-}$ and Bim (G154E/STA)-expressing cells possessed fewer lysosomes and they primarily clustered around the nucleus (Figure 4.5, panel A). To quantify lysosome number, we analyzed each image with ImageJ to determine the percent area occupied by lysosomes (Figure 4.5, panel B) and the total number of lysosomes (Figure 4.5, panel C) from each cell. In both cases, the quantified data suggested that Bim regulated lysosome number and positioning or trafficking in an LC8-dependent manner.

Finally, since the Bim-LC8 interaction mediated cathepsin trafficking and lysosome positioning, we speculated that these effects might be responsible for the inherent sensitivity of cells to heat shock-induced cell death. To address this hypothesis, we utilized the Ciliobrevin-D, the first identified small molecule inhibitor of dynein, which has been reported to effectively inhibit the dynein-dependent microtubule gliding [169]. Cells were pretreated with 100 μM of

Ciliobrevin-D for 2h, followed by heat shock treatment. Heat shock-induced cell death was then measured by AnnexinV/pi staining.

Remarkably, Ciliobrevin-D attenuated heat shock-induced cell death in wild-type, Bim (G154E), and Bim(ΔBH3)-expressing cells (Figure 3.6). However, no significant effect was observed in $Bim^{-/-}$ or Bim (G154E/STA)-expressing cells as they were directly resistant to cell death (Figure 4.6). This result supported the idea that inhibition of the dynein motor complex rendered cells resistant to heat by affecting lysosomal trafficking and that loss of Bim asserting phenocopied the effects of Ciliobrevin-D.

To summarize, in this chapter, we have shown that Bim mediates heat shock-induced LMP and cell death, independent of its BH3 domain and independent of Bax/Bak. Instead, Bim regulates LMP and cell death through its interaction with LC8 of the dynein motor complex that, in turn, by controls lysosomal trafficking and perhaps cathepsin secretion.

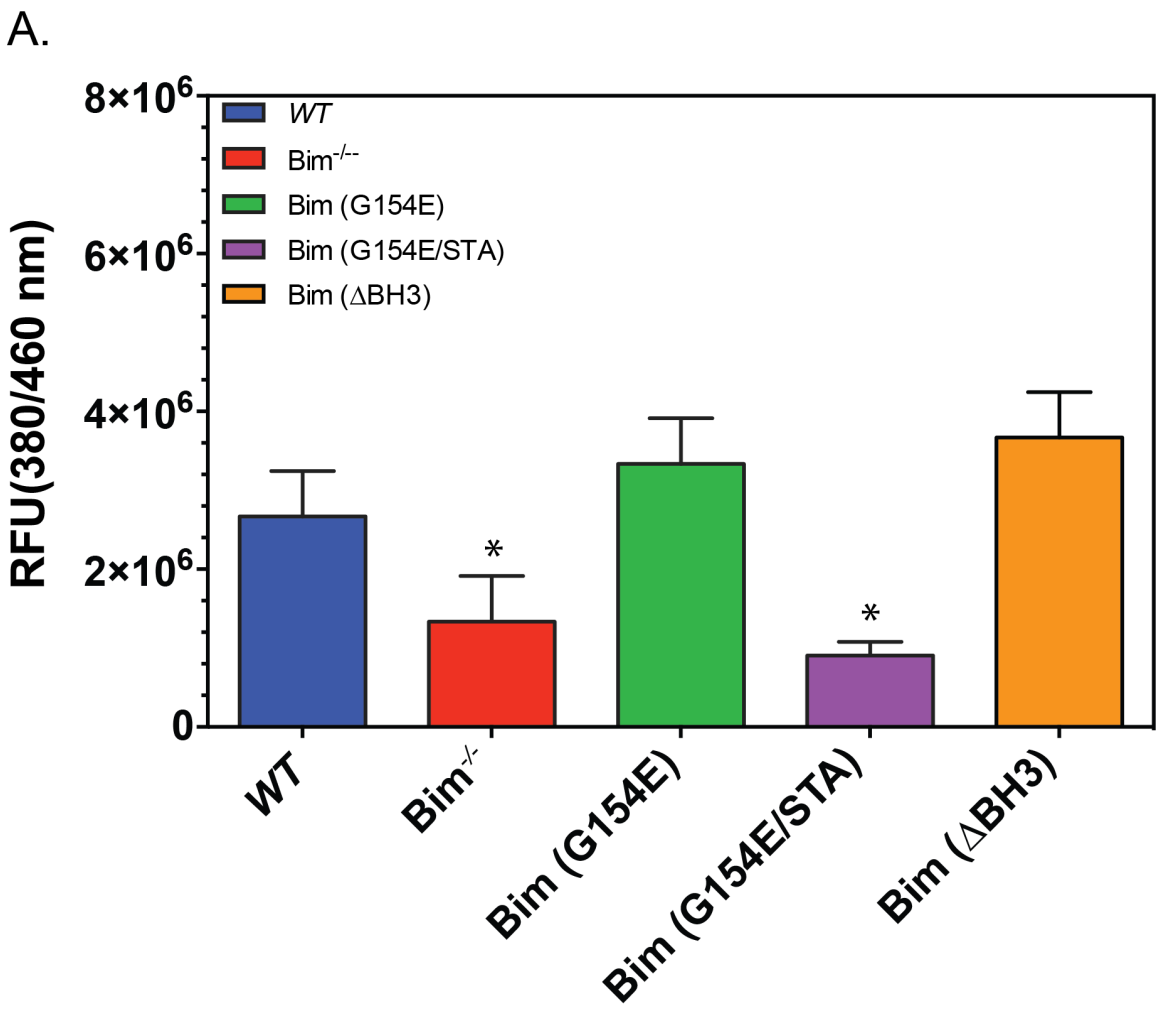

Figure 4.4. Bim and its interaction with LC8 is important for regulating the secretion of cathepsins.
Medium from vector control, Bim$^{-/-}$ re-expressing Bim (G154E), Bim (ΔBH3), or the LC8 binding mutant Bim (G154E/STA) was measured for cathepsin activity using the Omnicathepsin® fluorogenic substrate. (*: $P<0.05$)

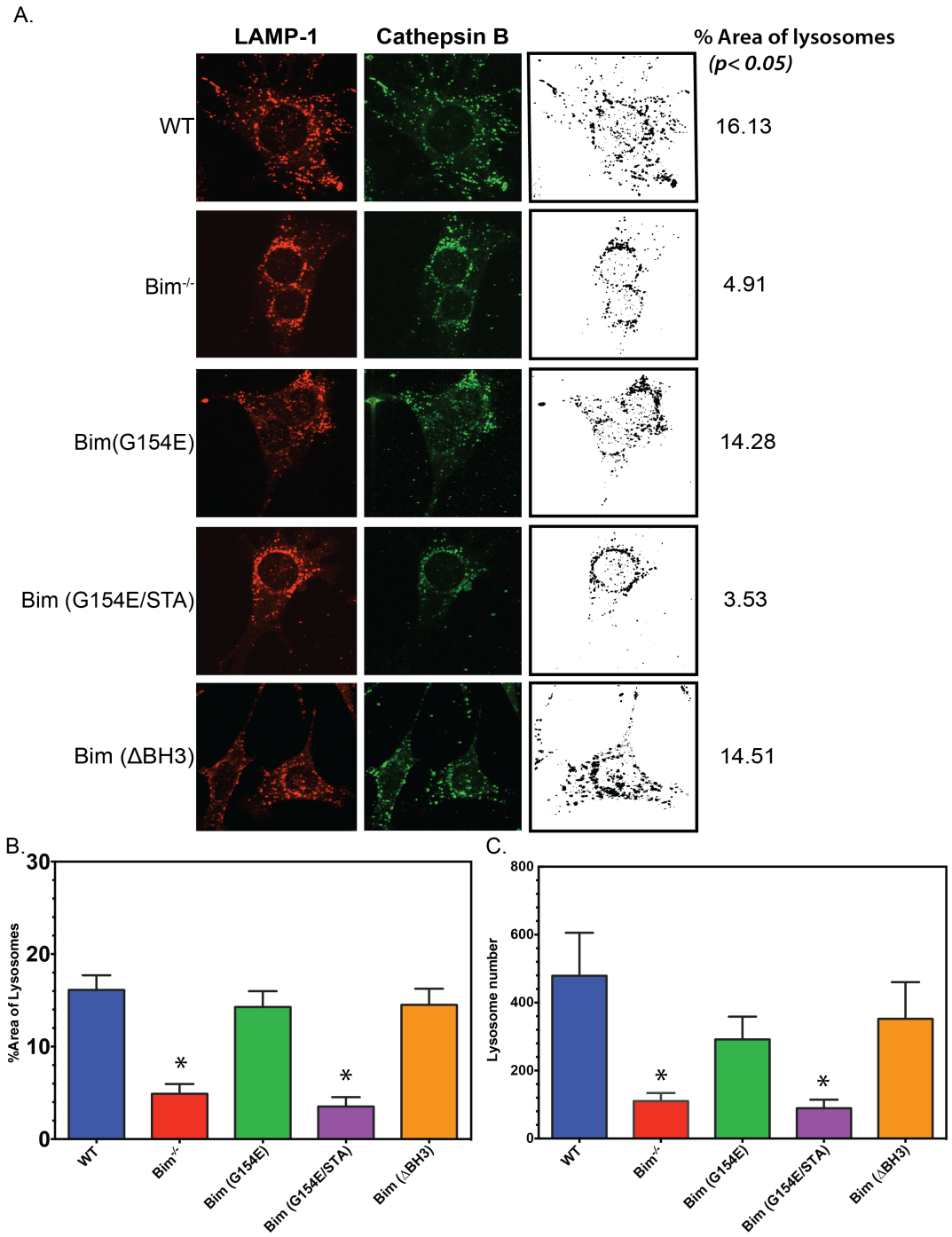

Figure 4.5. Bim regulates lysosome number and distribution through its interaction with LC8 of the dynein motor complex.

Vector control cells, and those stably expressing of Bim (G154E), Bim (ΔBH3), or Bim (G154E/STA) were stained for LAMP-1 and cathepsin B and imaged by confocal microscopy (panel A). The percent area occupied by lysosome and total lysosome number were quantified by ImageJ. (*: $P<0.05$)

A.

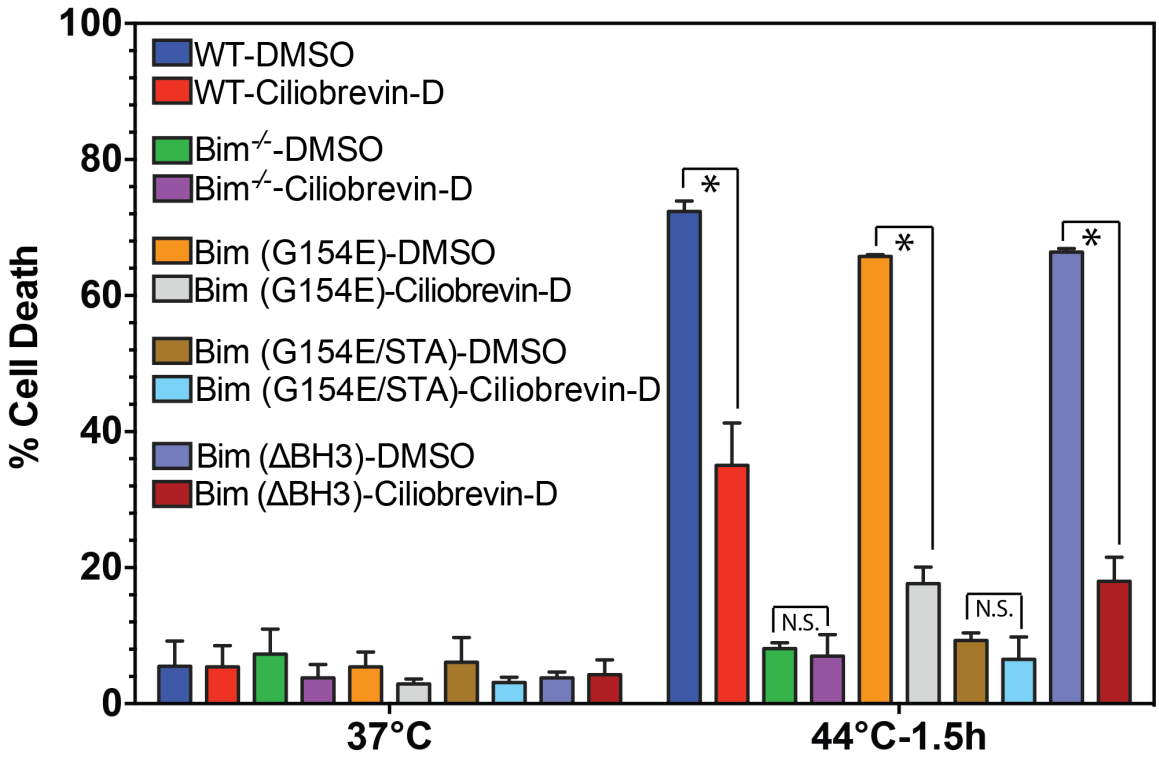

Figure 4.6. Heat shock-induced cell death can be attenuated by the dynein inhibitor Ciliobrevin-D.

Vector control cells, those stably expressed Bim (G154E), Bim (ΔBH3), or Bim (G154E/STA) were pretreated with either the vehicle control DMSO or the dynein inhibitor Ciliobrevin-D (100 μM for 2h), followed by exposure to heat shock (44°C for 1.5 h) in a humidified incubator (5% CO_2- 95% air). The cells were then transferred to a 37°C incubator and later collected for cell death measurement. (*: P<0.05; N.S.: No significant difference)

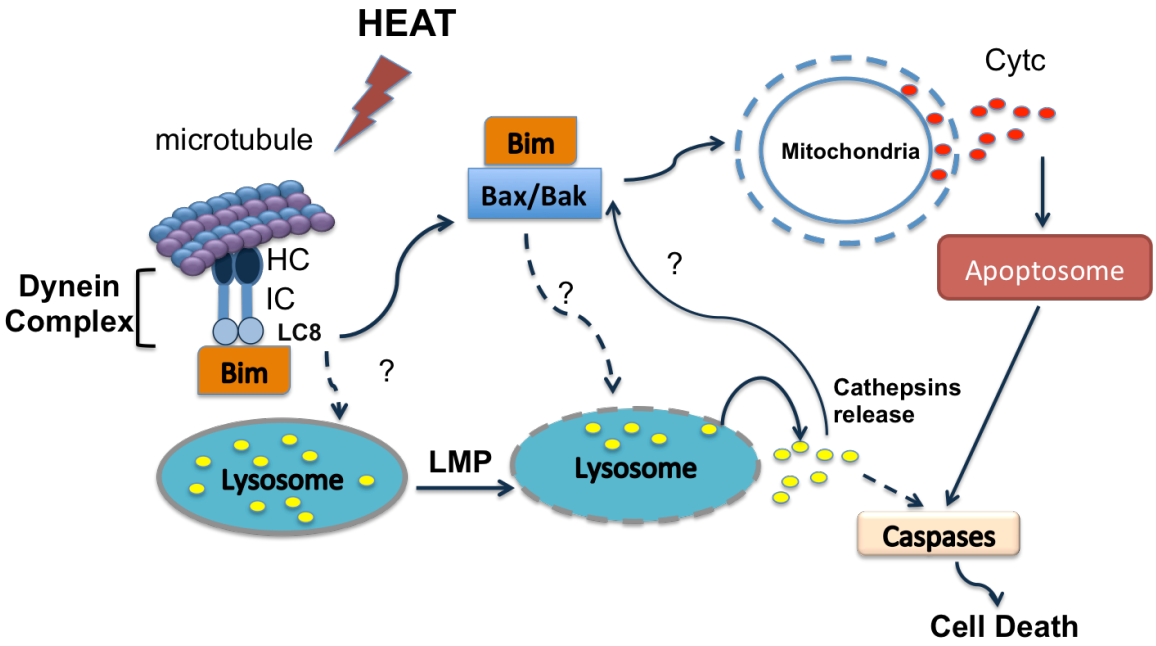

Figure 4.7. Bim-LC8 interaction mediates lysosomal cell death following heat shock.
Bim associates with the dynein motor complex to mediate heat shock-induced cell death. Bim's interaction with LC8 impacts lysosomal trafficking and is responsible for heat shock-induced LMP and cathepsin release. The released cathepsins subsequently activate down stream cell death pathways. Bim can also activate the canonical mitochondrial cell death pathway *via* direct activation of Bax/Bak, resulting in MOMP, cytochrome c release, and activation of Apaf-1 apoptosome.

4.3. Discussion

In Chapter 4, we examined how Bim regulates heat shock-induced LMP and cell death. Our data indicate that Bim does so in an LC8-dependent but BH3-independent manner. Beclin-1, the PI-3 kinase (PI3K) VPS34, and UVRAG form a complex that regulates autophagasome formation during autophagy, and it has recently been shown that Bim inhibits autophagy by sequestering Beclin-1 to microtubules *via* an interaction with LC8[52, 53]. Consequently, the authors found that Bim$^{-/-}$ cells exhibited higher levels of basal autophagy[53].

Interestingly, BCL-2 can bind to Beclin-1 through its BH3 domain and BH3-only proteins reportedly displace BCL-2 from Beclin-1, leading to formation of the Beclin-1/VPS34/UVRAG complex formation and an increase level of Autophagy. However, the interaction of Bim with LC8 and Beclin-1 appears not to involve Bim's BH3 domain, so that interplay between Bim, BCL-2, and Beclin-1 is complicated and remains unclear. Regardless, we found that Bim (G154E), which can bind to LC8 and Beclin-1 but not BCL-2 family members through its mutated BH3 domain, restored heat shock-induced LMP and cell death, whereas, Bim (G154E/STA), which can no longer bind to LC8 fails to do so. With regard to autophagy per se, we found that heat shock-induced lipidation of LC3 in wild-type and Bim$^{-/-}$ cells, as well as those expressing Bim (G154E) or Bim (G154E/STA). However, functional autophagy, as determined by turnover of p62,

was only observed in $Bim^{-/-}$ cells and those expressing Bim (G154E/STA), because they were the only cells resistant to heat shock-induced LMP. In other words, while all of the treated cell lines formed autophagosome, only those with intact lysosome could fuse with these autophagosome and degrade cellular cargo.

We have not yet determined if Bim's association with Beclin-1, VPS34, and UVRAG is required for heat shock-induced LMP. To address this, in the future we will utilize CRISPR-Cas9 technology to knockout endogenous Beclin-1, VPS34, or UVRAG. However, for Beclin-1 and VPS34, this may be lethal to the cells. Beclin-1 knockout mice die early in development[170], in contrast to the knockout of other autophagy genes indicating that it plays important roles in cellular events other than autophagy. Therefore, we may be forced to transiently overexpress a dominant negative of Beclin-1 that cannot interact with LC8 or perhaps stably express this mutant and then knockout the endogenous Beclin-1.

As for VPS34, we can utilize the pharmacological inhibitor VPS34-IN1, which is reportedly as a specific VPS34 inhibitor[171]. Preliminary data with this inhibitor suggests that it reduces heat shock-induced cell death (data not shown). In using these various approaches, we will rule in or out whether the role of the Beclin-1/VPS34 complex in regulating Bim-mediated heat shock-induced LMP and cell death.

Our data with ATG5$^{-/-}$ and ATG16L1$^{-/-}$ cells suggests that loss of autophagy does not dramatically increase cell death resulting from heat shock. Thus, we currently speculate that autophagy does not contribute to the resistance of Bim$^{-/-}$ cells to heat shock. However, to confirm this interpretation, we will knockout endogenous ATG5 in Bim$^{-/-}$ cells using CRISPR-Cas9. If *Bim*$^{-/-}$ /ATG5$^{-/-}$ DKO cells remain fully resistant from heat shock-induced LMP and cell death, it will suggest that autophagy is not responsible for the resistance of Bim$^{-/-}$ cells to heat shock.

In Chapter 3, the role of intracellular and extracellular cathepsins in heat shock-induced lysosomal cell death was discussed. Our data in Chapter 4 suggests that Bim mediates heat shock-induced LMP and cell death through its effects on lysosomal trafficking and the secretion of cathepsins. The dynein inhibitor Ciliobrevin-D[169, 172], phenocopied the loss of Bim, at least in turns of mediating resistance to heat shock. Ciliobrevin-D protected ~ 50% of wild-type cells from heat shock-induced cell death. However, we are not sure exactly how Ciliobrevin-D blocks cell death. Based on our analysis of Bim$^{-/-}$ cells and those expressing Bim mutants (Figure 4.5 and 4.6), we speculate that lysosomal positioning is correlated to heat shock-induced LMP and cell death, and that those lysosomes distributed to the cell periphery are more prone to heat shock-induced LMP and serve them of extracellular cathepsins through lysosomal

exocytosis. Notably, the dynein intermediate chain is important for the binding of LC8 to the dynein motor complex[50, 173], and recently, a dimeric LC8 traps has been developed that sequesters LC8 and promotes the rapid movement of lysosomes to the periphery[174]. In preliminary data, we found that this LC8 trap restores sensitivity of Bim$^{-/-}$ to heat shock-induced cell death. To evaluate this hypothesis, we will overexpress kinesin regulator protein, such as KIF2a and Arl8, which drive plus end movement of organelles to the periphary[86, 175, 176], or RILP which facilitates minus end movement to the perinuclear area[81-83, 87, 154, 172, 173, 177].

Of course, following each of these treatments, we will need to determine lysosome number, position, and cathepsin secretion, just as we have already done for Bim$^{-/-}$ cells and those reexpressing Bim mutants. Previously in Chapter 3, we showed that CRISPR-Cas9 knockout of Bim in the Bax$^{-/-}$/Bak$^{-/-}$ DKO cells confers further resistance to heat shock-induced LMP and cell death. We will need to similarly examine theses cells for changes in lysosome number and distribution.

Finally, while peripheral localization of lysosomes may sensitize them to heat-mediated LMP, increased cathepsin secretion could be also important in amplifying the cell death signal. Secreted cathepsins as known to digest extracellular matrix during the cancer metastasis, cancer promotion, or induction

of anoikis[5, 12, 16, 68, 69, 120]. It's likely that lysosome positioning is affecting cathepsin secretion, resulting in increased break down of extracellular matrix, which may be inherently enhanced by heat shock. In order to address this hypothesis, we will utilize the pharmacological inhibitor, vacuolin-1, which reportedly inhibit Ca^{2+}-dependent lysosomal exocytosis[178]. In using this inhibitor, the secretion of cathepsins should be blocked, and then we can evaluate this effect on heat shock-induced LMP and cell death.

Previous work suggests that synaptotagmin VII (Syt VII) regulates Ca^{2+}-triggered exocytosis, plasma membrane repair, regulates synaptic vesicle exocytosis [80]. Therefore, we also plan to knock out Syt VII. This can be done in cells expressing LC8-Trap, Arl8, and KIF2a, so that, we can examine cells possess peripherally-located lysosomes but are deficient in cathepsin secretion. This will let us know whether it is LMP and the release of intracellular cathepsins, or an increased in the secretion of extracellular cathepsins that is most important in mediating heat shock-induced cell death.

Chapter 5. Conclusion

In this dissertation, I have demonstrated that heat shock induces cell death through both Bax/Bak-dependent and -independent pathways and that Bim is essential for the activation of both. Bim can directly activate Bax/Bak, resulting in MOMP and the release of cytochrome c, followed by apoptosome formation and the sequential activation of caspases-9 and -3. However, this pathway is only partially responsible for cell death at lower exposure of heat. Bim also mediates LMP independent of Bax/Bak following both short and prolonged exposures to heat shocks. This lysosomal-mediated cell death pathway involves LMP and the release of cathepsins into cytoplasm. The released cathepsins, particularly cathepsin L, cleaves Bid, which in turn activates Bax/Bak to engage the aforementioned mitochondrial pathway; or cleave other substrates that result in cell death. Notably, this lysosomal-mediated cell death can be attenuated by overexpression of CSTB, which suggests that cysteine cathepsins play an important role in mediating cell death, and instead, cathepsin L appears to be essential for heat shock-induced LMP (i.e. its own release) and cell death. Heat shock-induced cell death is almost completely blocked in cathpeisnL$^{-/-}$ cells and it restored upon reintroduction into cells. Since cathepsin L is one of the most secreted cathepsins, we questioned whether cathepsins might initiate cell non-autonomous death following heat shock and induced expression of secreted

cystatin C inhibitor into the media or addition of recombinant CSTB partially suppressed cell death.

We further examined how Bim mediated LMP following heat shock. We found that Bim does so independently of its BH3 domain, which is remarkable given all previous literatures on Bim has shown its BH3 domain to be essential for cell death in response to other stimuli. Both Bim (G154E) and Bim (ΔBH3) -expressing cells restored heat shock-induced LMP and cell death upon reintroduction into $Bim^{-/-}$ cells. By contrast, a Bim mutant (BimG154E/STA) that disrupts its interaction with the dynein light chain LC8, failed to induce LMP and cell death. Therefore, these results suggest that, rather than utilizing its BH3 domain to mediate cell death, Bim requires its interaction with the dynein motor complex to induce LMP and cell death following heat shock.

Bim and its interaction with LC8 has been reported to negatively regulate autophagy by sequestering Beclin-1 to the dynein motor complex. Thus, we initially suspected that loss of Bim might suppress heat shock-induced cell death by increasing autophagy. $Bim^{-/-}$ and Bim (G154E/STA)-expressing cells exhibited increased autophagy following heat shock, as indicated by an increase in LC3 lipidation and the turnover of p62; however, wild-type and Bim (G154E) expressing cells failed to undergo autophagy because they underwent LMP, and thus could not fuse with autophagosomes. To our surprise, however, $ATG5^{-/-}$ and

ATG16L1$^{-/-}$ cells showed no significant increase in heat shock-induced cell death compared to wild-type cells, indicating that autophagy was not responsible for the pro-survival signals afforded by the loss of Bim.

Up until now, the prevailing wisdom has been that LC8 sequestration of Bim was recently for the purpose of preventing Bim from engaging BCL-2 family member and inducing MOMP, and more recently for recruiting Beclin-1 resulting in suppression of autophagy. However, the dynein motor complex, along with kinesin, are important in organelle transport and sorting by trafficking vesicles along microtubules. Therefore, we speculated that Bim might also be important in the regulating of endolysosomal trafficking and thus lysosomal biogenesis and positioning.

As predicted, we found that Bim$^{-/-}$ and Bim (G154E/STA)-expressing cells possessed fewer lysosomes, most of which clustered around the nucleus. By contrast, wild-type, Bim (G154E), and Bim (ΔBH3) contained a normal number of lysosomes, which were distributed throughout the cytosol. More excitingly, the distribution of lysosomes appeared to correlate with the presence of extracellular cathepsin activity. Wild-type, Bim (G154E), and Bim (ΔBH3) had much higher levels of extracellular cathepsins activity, while Bim$^{-/-}$ and Bim (G154E/STA)-expressing cells exhibited significantly lower levels of extracellular cathepsin activity. This result linked Bim-LC8 interactions with lysosome number,

positioning, and cathepsins secretion. Especially interesting, the effects of Bim on lysosomes correlated with heat shock-induced LMP and cell death. Using the dynein inhibitor Ciliobrevin-D, which inhibits microtubule gliding, we could similarly suppress heat shock-induced cell death in wild-type cells. Collectively, these results led us to speculate that Bim functioned as an antagonist of dynein motor complex, regulating lysosomal distribution and cathepsin trafficking through its interaction with LC8. This hypothesis was further supported by the ability of an LC8-trap to sensitize $Bim^{-/-}$ cells to heat shock-induced cell death.

Thus, Bim mediates LMP and cell death through three different but highly interrelated signaling pathways following heat shock. Bim mediates Bax/Bak-dependent mitochondrial cell death, Bax/Bax-independent LMP and cell death, and Bim-LC8 regulated lysosomal distribution and cathepsin trafficking. While $Bax^{-/-}$/$Bak^{-/-}$ DKO cells were partially resistant to cell death following heat shock, deletion of Bim rendered cells completely resistant to heat shock and reintroduction of BH3 mutant, Bim (G154E) restored 100% of cell death. This raised the question of how Bim (G154E) accounted for the ~50% of cell death not mediated by Bax and Bak? As we discussed in Chapter 3, once released, cathepsins can cleave Bid, which can then activate Bax/Bak and subsequently amplify the cell death signal by inducing MOMP. Thus, tBid is likely responsible for inducing the Bax/Bak-dependent cell death in Bim (G154E)-expressing cells. To confirm this hypothesis, in the future, we will knockout endogenous Bid in

using CRISPR-Cas9 technology in Bim (G154E) expressing cells. If loss of Bid attenuates ~ 50% of cell death in these cells, like that observed in $Bax^{-/-}/Bak^{-/-}$ DKO cells, then we will know that Bid is playing an important role in amplifying the cell death signal following heat shock.

Cathepsin L-deficient cells were also completely resistant to heat shock-induced LMP and cell death. We speculate that cathepsin L is acting as the apical protease, that once released, activates Bid and cleaves other critical substrates. To confirm this hypothesis, we could generate a mutant form of Bid that is resistant to cleavage by cathepsin L and reintroduce it into $Bid^{-/-}$ cells to see if it only partially restores cell death in response to heat shock.

In Chapter 3, we demonstrated that heat shock-induced LMP began earlier than MOMP. However, the window between two events was narrow, and we have not entirely ruled out the possibility that both events may begin simultaneously following heat shock. To address this question, we plan to stably co-express cytochrome c-GFP and cathepsin L-mcherry in cells and simultaneously monitor both LMP and MOMP using time-lapse microscopy following heat shock. Using this approach, we can examine, on a single cell basis, both LMP and MOMP to determine the precise time frame over which each event occurs.

In Chapter 4, we demonstrated that Bim could mediate LMP, independent of its BH3 domain. However, since BCL-2 is overexpressed in many cancers, it is possible that BCL-2 could suppress heat shock-induced apoptosis by binding to Bim and disrupting its regulation of the dynein motor complex. To further investigate this angle, we will over express BCL-2 in both $Bax^{-/-}$/$Bak^{-/-}$ DKO cells (which contain wild-type Bim) and Bim (G154E)-expressing cells. If expressing BCL-2 inhibits cells death in $Bax^{-/-}$/$Bak^{-/-}$ DKO cells but not in Bim (G154E)-expressing cells, it will suggest that BCL-2 can suppress heat shock-induced LMP and cell death by binding to Bim and disrupting its noncanonical control of lysosomal trafficking and cathepsins secretion.

As already discussed, to confirm that Bim-LC8 interactions regulate lysosomal distribution and cathepsin trafficking, we will overexpress both dynein (RILP) and kinesin (Arl8 and KIF2) effectors and then examine how cells response to heat shock. If cells with peripherally distributed lysosomes become sensitive to heat shock-induced LMP an exhibit higher levels of extracellular cathepsin activity, it will suggest that these events are essential for heat shock-induced cell death. Vaculoin-1 and overexpression of Syt VII (synaptotagmin VII) that inhibit secretion should help to answer this question. Hopefully, we will be able to as certain the extent to which intracellular and extracellular cathepsins contributes to cell death and whether the latter is cell autonomous or non cell autonomous.

Overall, our study reveals a highly novel mechanism of Bim-mediated LMP and cell death following heat shock. With a better understanding of the precise mechanism(s) of heat shock-induced LMP and cell death, we hope to evaluate the potential pathways of how Bim regulates dynein motor complex and how secreted/released cathepsins L mediates cell death following heat shock in the future. For instance, cancer cells that possess lower levels of Bim, either due to deletion or epigenetic silence may not respond well to standard heat shock therapy. However, Bim mimetics or LC8-traps may be able to restore sensitivity of Bim-deficient cancer cells to heat shock.

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Vita

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